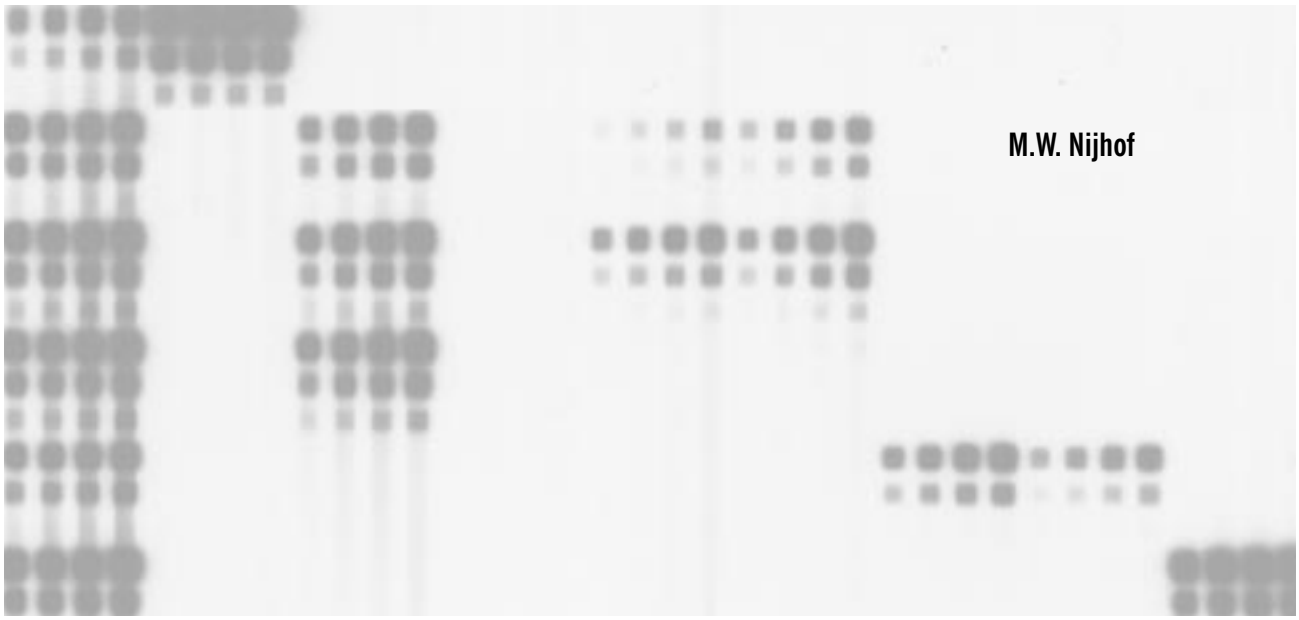
Musculoskeletal infections Developments in Diagnosis and Treatment



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MUSCULOSKELETAL INFECTIONS

Developments in Diagnosis and Treatment

INFECTIES VAN HET STEUN- EN BEWEGINGSAPPARAAT

Ontwikkelingen in Diagnostiek en Behandeling

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van
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Marc Willibrordus Nijhof

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- Nijhof MW, Oyen WJG, van Kampen A, Claessens RAMJ, van der Meer JWM, Corstens FHM. Evaluation of infections of the locomotor system with indium-111- labeled human IgG scintigraphy. J Nucl Med 1997;38:1300-1305.
- Nijhof MW, Oyen WJG, van Kampen A, Claessens RAMJ, van der Meer JWM, Corstens FHM. Hip and knee arthroplasty infection. In-111-IgG scintigraphy in 102 cases. Acta Orthop Scand 1997;68:332-336.
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- Nijhof MW, Schot CS, Wilbrink B, Vogely HC, Fleer A, Verbout AJ, Dhert WJA, Schouls LM. Molecular diagnosis of musculoskeletal infection using PCR and reverse line blot. Submitted for publication.
- Nijhof MW, Fleer A, Dhert WJA, Verbout AJ. Review on antimicrobial-loaded carriers in musculoskeletal infection. In manuscript.
- Nijhof MW, Dhert WJA, Tilman PBJ, Fleer A, Verbout AJ. Release of tobramycin from tobramycin-containing bone cement in bone and serum of rabbits. J Mater Sci: Mater Med 1997;8:799-802.
- Nijhof MW, Dhert WJA, Fleer A, Vogely HC, Verbout AJ. Prophylaxis of implant-related staphylococcal infections using tobramycin-containing bone cement. J Biomed Mater Res 2000;52:754-761.
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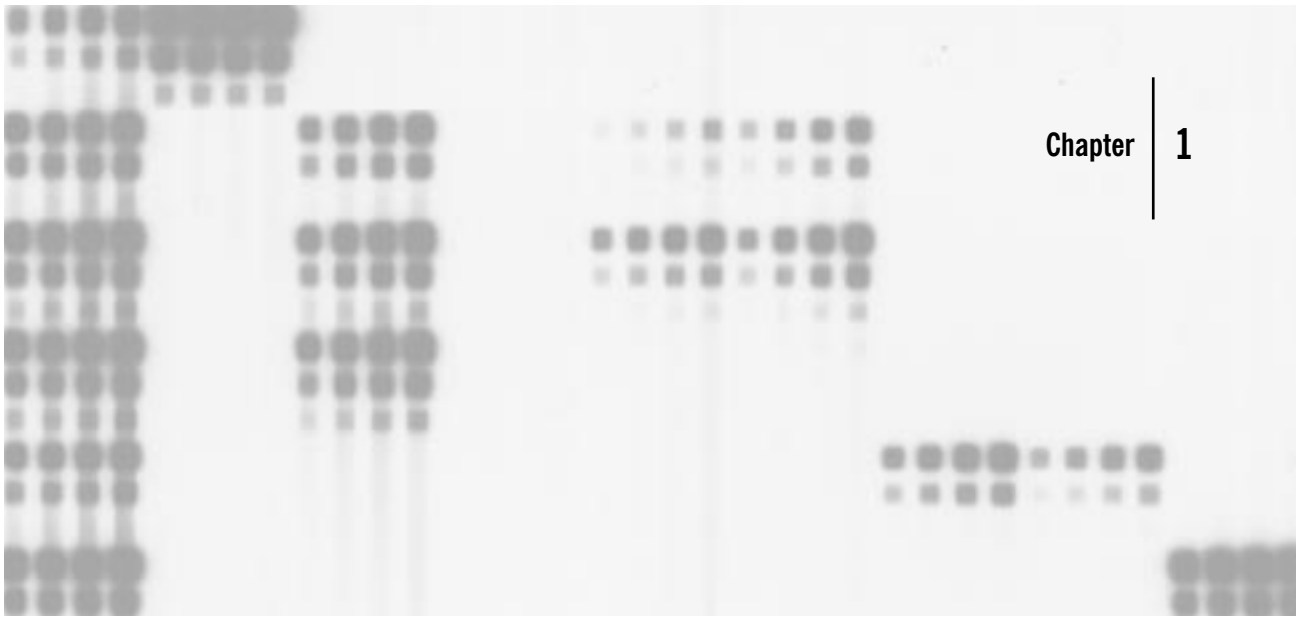
- Nijhof MW, Hardus KE, Fleer A, Vogely HC, Schouls LM, Verbout AJ, Dhert WJA. Tobramycin-containing bone cement and systemic cefazolin in a one-stage revision. Treatment of infection in a rabbit model. Submitted for publication.

General introduction



I Introduction to musculoskeletal infection

Infections of the musculoskeletal system , or orthopaedic infections are terms that are used in literature to lump together infections of bone, cartilage and soft tissues, like muscle, tendon and synovium. Such infections, whether these occur as a complication of surgery, mechanical injury or due to other causes, can seriously affect the quality of life of patients. The major impact of musculoskeletal infection on healthcare, also in terms of financial costs, has led to great scientific effort to improve the management of these infections, by studying their nature and strategies for prevention and treatment. Nevertheless, many strategies related to this complication in orthopaedics can and need to be further optimized. This is partially due to the fact that medicine is evolving and new techniques and products for medical use have to be and are constantly being evaluated. But another major factor that makes research difficult in the field of musculoskeletal infection - of which osteomyelitis and septic arthritis are the main representatives - is the multifactorial aspect of these diseases. Since this thesis is based on studies of the treatment and prevention of orthopaedic infections, including the diagnostic approach, it is important to outline the multifactorial aspect of the problem, that may determine the outcome of such studies. This chapter gives an overview of several aspects of musculoskeletal infections, and discusses their relevance and implications for research on the management of these diseases.

I Infection mechanism

The spectrum of associated pathogens of musculoskeletal infection changes with host age, infection site, entrance route, underlying systemic or local pathologic conditions and the presence of foreign bodies, such as a joint prosthesis and osteosynthesis material. All these host-related factors that may decrease the ability of the patient s immune system to fight the infection, can play a role in the initial stages of development of an infection, but can also determine to some extent the course and outcome of the infection. A variety of characteristics of the microorganisms are known to act/counteract with these host factors. These include the genetically driven assembly of virulence substances like adherence receptors and toxins, and the capability to pheno-

typically alter the growth rate.
The response of the musculoskeletal tissues to the presence of these bacteria can show a great variation, depending on the host and microbial factors mentioned above. Some pathogens and infection sites are known to predispose for a fulminant onset of the infection, resulting eventually in a full-blown pyogenic infection, such as in the case of *Staphylococcus aureus* septic arthritis. Other species or even strains of the same species may, in contrast, cause an indolent osteomyelitis at for instance the site of an orthopaedic implant, presenting with few clinical or laboratory signs of an inflammatory response to these pathogens.
The multitude of factors related to the prevention of osteomyelitis reflects the multifactorial aspect of musculoskeletal infection. To illustrate this, the variety of risk factors that has been associated with musculoskeletal infection is discussed below, and the prophylactic measures which can be undertaken to minimize these risks.

I Risk factors for musculoskeletal infection

The concept of the triad of bacteria, host and wound can be used to visualize the relationship between the risk-factors and to differentiate the prophylactic approach [Hanssen, 1997]. In Table 1, an overview of the many risk factors for orthopaedic surgical infections as reported in literature is presented, and categorized by effect on the three components of the triad. Clearly, these components and factors are closely related to each other and some overlap exists: Decreased wound healing itself can affect the number of bacteria in a wound. Malnutrition, diabetes mellitus and rheumatoid arthritis have been correlated with not only a delayed wound healing, but also with a decrease in immunocompetence due to lower lymphocyte counts and decreased neutrophil function respectively [England, 1990, Gherini, 1993, Jensen, 1982, Wilson, 1990b]. Both a delayed wound healing and a decrease in immunocompetence may attribute to a higher risk of infection. Furthermore, ulcers of the skin and recurrent breakdown of the skin may pose the rheumatoid and diabetic patient at risk for delayed haematogenous infection after arthroplasty [Papagelopoulos, 1996, Wilson, 1990b]. The relationship between the risk factors makes it difficult to point out single risk factors and to estimate their potential to contribute to infection rate. Indeed, some studies could demon-

strate a significant difference among various risk factors, whereas others could not. Multicenter prospective studies should provide more extensive data to rationalize our choices in patient care.

Certainly, these studies have underscored the importance of identifying, and, when possible, eliminating any risk factor for infection in a patient undergoing arthroplasty surgery. However, full proof of the efficacy of some prophylactic measures may be marginal [Gillespie, 1997]. Furthermore, questions of generalizability of some results remain, and in addition it is difficult to estimate the additional benefit of each prophylactic technique when used in combination with others. The latter was illustrated by a study of Lidwell *et al.* in which he showed that ultraclean air could positively affect the infection rate after surgery [Lidwell, 1982]. However, these and other authors recognized the lack of randomization on use of antibiotics in that study, and it appeared that the same effect could be achieved without ultraclean air when antibiotics were used for infection prophylaxis [Garvin, 1995, Hanssen, 1997, Lidwell, 1986].

In addition to existing prophylactic measures, the design of new techniques and materials to optimize infection prophylaxis in orthopaedics is ongoing.

Table 1. Summary of risk factors of orthopaedic surgical infection, based on the Bacteria, host and wound triad.

Bacteria - factors that influence the number or virulence of bacteria introduced into a wound	
Foreign bodies (biomaterials)	Joint prosthesis or osteosynthesis material, especially when previously infected [Cherney, 1983, Gristina, 1985, Naylor, 1990, Poss, 1984, Schmalzried, 1992]
	Bone graft [Schutzer, 1988]
Operating room	Number and traffic of theatre personnel [Borst, 1986, Nelson, 1987, Ritter, 1987]
	Operation time [Chan, 1998, Charnley, 1972, Fitzgerald, 1977]
	Airflow in theatre [Brady, 1975, Charnley, 1972, Fitzgerald, 1992, Glynn, 1983, Hanssen, 1994, Lidwell, 1986, Lidwell, 1982, Marotte, 1987, Nelson, 1980, Salvati, 1982b]
	Early shaving of the operation site [Mishriki, 1990, Seropian, 1971]
	Permeable surgical dress and drapes [Blomgren, 1990, French, 1976, Johnston, 1987, Whyte, 1983]
	Use of a suction tip [Greenough, 1986, Meals, 1978, Strange-Vognsen, 1988]

Continued

Host - factors that influence the host's capability to response to the infection	
Integrity of skin and mucosa distant from operation site (bacteremia)	Vascular catheter [Friedman, 1988, Wilkins, 1990]
	Urinary catheters [Maderazo, 1988, Michelson, 1988, Ritter, 1989, Stamm, 1991, Wroblewski, 1980]
	Major dental procedure [Drangsholt, 1998, Sandhu, 1997, Segreti, 1999, Thyne, 1991, Waldman, 1997]
	Decubitus ulcer [Deacon, 1996, Wilson, 1990b]
	Psoriasis [Beyer, 1991, Menon, 1983b]
Hospital stay	Time in hospital [Cruse, 1973]
	Time on intensive care [Vincent, 1995]
Systemic disorders or altered general state of health	Rheumatoid arthritis [Deacon, 1996, Poss, 1984, Wilson, 1990b]
	Insulin-dependent (type 1) diabetes mellitus [England, 1990, Menon, 1983a, Papagelopoulos, 1996]
	Hemophilia [Goldberg, 1981, Lachiewicz, 1996, Luck, 1989, McCollough, 1979]
	Malnourishment [Gherini, 1993, Greene, 1991, Jensen, 1982, Puskarich, 1990, Smith, 1991]
	Use of immunosuppressive drugs [Wilson, 1990b]
	Obesity [Stern, 1990, Wilson, 1990b]
	Allogeneic blood transfusion [Fernandez, 1992, Murphy, 1991]
Wound - Factors that influence wound healing and viability	
Compromized bone or soft tissue	Devascularization, dead space and hematoma [Fitzgerald, 1977, Glynn, 1983, Nelson, 1987, Nelson, 1980]
	Previous operation on the affected joint [Fitzgerald, 1977, Nelson, 1980, Rand, 1989, Surin, 1983, Windsor, 1990]

For instance, it can be expected that a wider range of antibiotics in various release systems or carriers will become available in the future. Biodegradable materials are increasingly used for this purpose. Obviously, studies that evalu-

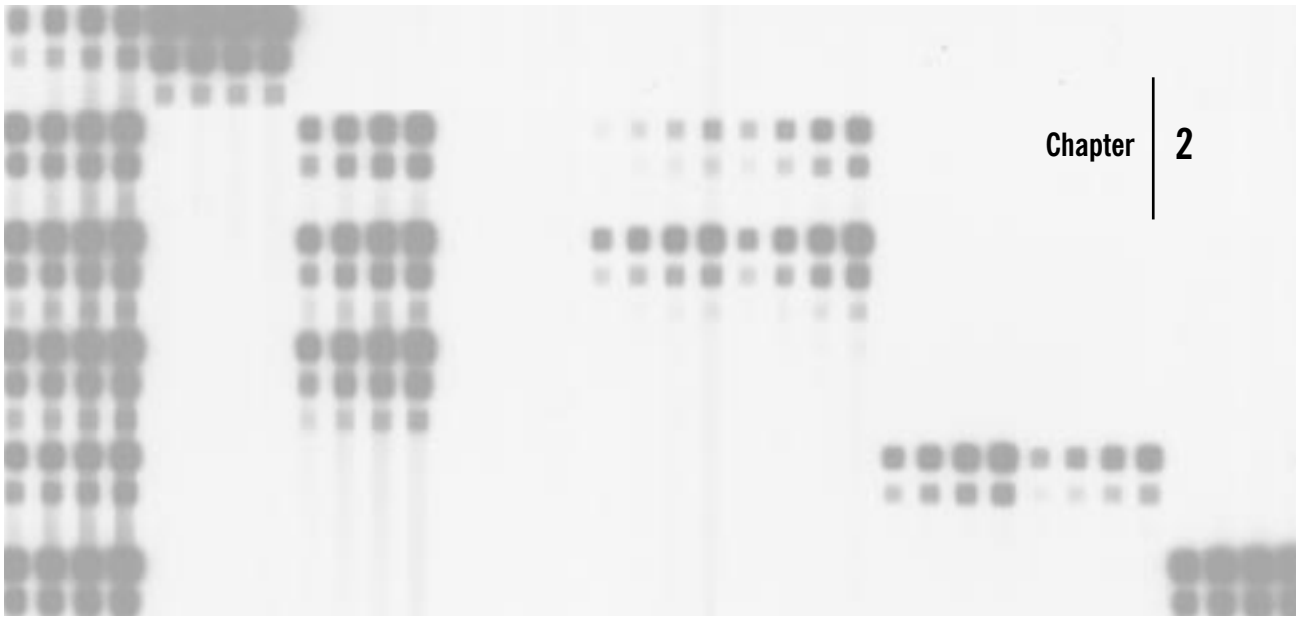
ate release characteristics of antibiotic-loaded carriers should reveal their potency to be of use in infection management. Likewise, the biocompatibility of such new (biodegradable) implants, determined by their composition, is important. New or modified antibiotic-containing implants, to be used in the management of (orthopaedic) infection, should indeed be non-toxic and non-allergenic. In addition, inflammatory components should not be present, which may otherwise influence the healing of tissue defects, especially in areas of infection. Thus, not only the release characteristics and eventually the degradation rate of an antibiotic-loaded carrier, but also its biocompatibility are important aspects in the design of such an implant [Garvin, 1994].

Subsequently, *in vitro* and experimental efficacy tests may demonstrate their bacterial inhibitory properties. Finally, some of these implants will be studied clinically, and approved for clinical use in the management of orthopaedic infection. Continuous monitoring of such biomaterials, or any other method used clinically for the management of orthopaedic infection, is vital for an adequate evaluation of their efficacy and safety. Governmental bodies like the Federal Drug Agency and (inter-)national societies for research in orthopaedic or biomaterials contribute to this process of objective evaluation of new and existing techniques. Orthopaedic surgeons play an important role in this process because they can judge clinical outcome in their patients. Especially when they participate in ventures such as the Scandinavian or other arthroplasty registries, clinical data become available for objective evaluation [Berry, 1997, Fender, 1997, Havelin, 2000, Herberts, 2000, Malchau, 2000, Paavolainen, 1991, Rothwell, 1999, Sarungi, 2000]. However, as a consequence of the many variables in orthopaedic infections and related risk factors, the approach to infection prophylaxis in orthopaedics is and should be multidisciplinary. Not only the orthopaedic surgeon is involved in this process, but also other medical specialties, theatre staff, personnel on the intensive care and hospital wards, the microbiologist, hospital committees for hygiene and infection prevention, and the general practitioner. As a result of an increased awareness of risk factors and development of new prophylactic techniques, infection rates should decrease. Indeed, recent data show that infection incidence has decreased over the years, presumably due to the many prophylactic measures that are now widely applied [Malchau, 2000]. The latest annual report of the Swedish National Hip Arthroplasty Registry states that the 10-years cumulative frequency of revision due to infection of total hip prostheses inserted in 1979 was 0.8-0.9%, and this had decreased to 0.3% for hip prostheses that were implanted in 1988 [Malchau, 2000].

I Outline of this thesis

This thesis represents the multifactorial approach to the clinical problem of musculoskeletal infection. Management of this infection does not only include prevention, but also deals with difficulties in diagnosis and treatment of the disease. Certainly, the fact that many diagnostic modalities and treatment options for musculoskeletal infection exist, indicates that none of these are fully accurate or effective in all patients [Elgazzar, 1995]. Therefore, the search for new and better tools for diagnosis and treatment of orthopaedic infection is ongoing, just as it is for prophylaxis of it. In **part I** of this thesis, new techniques have been investigated for the diagnosis of musculoskeletal infection. These include the use of radiopharmaceuticals for the imaging of musculoskeletal infection, and a new molecular technique, which might enable the detection of bacterial DNA in tissue samples of patients, suspected for infection. **Part II** of this thesis consists of experimental studies investigating a new pre-mixed tobramycin-containing bone cement. The *in vivo* release of tobramycin and the efficacy of this cement in prevention and treatment of prosthesis-related infection were studied.

Introduction to new diagnostic techniques



Chapter 2

I Introduction

The management of infection in orthopaedics, such as osteomyelitis, soft tissue infection, infectious arthritis and arthroplasty infection, remains a challenging clinical problem. A high index of suspicion, early intervention, and aggressive treatment are important in order to clear the infection [Tetsworth, 1999]. Early intervention requires an early diagnosis of infection. Aggressive treatment can not be complete without the full knowledge of the pathogen, severity and extent of the infectious process. Especially in chronic osteomyelitis and in arthroplasty infection, problems in the diagnosis of infection are frequently encountered. In contrast to a full-blown acute osteomyelitis, symptoms and signs of infection may be minimal in chronic osteomyelitis. Becker described how the pathogenesis of diabetic foot can contribute to difficulties in diagnosis of chronic osteomyelitis in these patients [Becker, 1999]: Pedal osteomyelitis in the diabetic foot often results from continuous spread from skin ulcers, secondary to neuropathy and macroangiopathy. Inhibition of bone resorption, periosteal new bone formation, and healing due to ischemia may lead to atypical changes of osteomyelitis. The sequela osteomyelitic bone resorption, pathological fracture, diminished intraosseous blood vascularity, and osteonecrosis can precede chronic osteomyelitis in the diabetic foot. Chronic pedal osteomyelitis combined with the absence of pain and proprioceptive sensation leads to pathologic fractures, bone sclerosis and subluxations, findings that can mimic pure neuroarthropathy. The absence of fever, bacteremia and elevated erythrocyte sedimentation rate may hamper the early diagnosis of diabetic pedal osteomyelitis even more. Undiagnosed, the ongoing osteomyelitic process in these and other patients with chronic osteomyelitis can result in considerable disability and morbidity.

In arthroplasty, two major complications - loosening and infection of the implant - can sometimes be difficult to distinguish. Both can present themselves clinically as painful joints, with equivocal or nonconclusive follow-up. Furthermore, growth characteristics of bacteria embedded on the surface of a foreign body can change, contributing even more to the insidious nature of implant infections. When the causative organism cannot be detected and/or the spread of infection cannot be visualized, the surgical and/or antibiotic treatment cannot be facilitated and optimized. It can therefore be concluded that diagnosis of bone and prosthesis infections remains a difficult challenge, and many have focused and will focus on improvement of diagnosis. During

the last years, improvements have been made in the use of nuclear medicine techniques, and in molecular biological techniques. In **part I** of this thesis, some of these new modalities in nuclear medicine for the imaging of orthopaedic infections were explored, as well as new tools in molecular biology for the detection of the pathogens of these infections.

I Scintigraphic imaging of musculoskeletal infection

For imaging of either implant related or other forms of musculoskeletal infections, different diagnostic modalities are available, including conventional radiography, computed tomography, magnetic resonance imaging and scintigraphic techniques. On radiographs, local nonspecific (swelling, thickening or elevation of the periosteal and cortical erosions) and more specific (sequester, Brodie s abscess) bone and soft tissue responses to infection can be seen. It can take more than two weeks for these changes to become visible on radiographs [Al-Sheikh, 1985, Norden, 1980]. Anatomic changes due to the infection have to be present as well in order to be visualized by computed tomography. A previously distorted anatomy may hinder image interpretation. The direction of the CT-plane is usually transversal, which makes it difficult to determine the extent of the infection [Wegener, 1991]. Magnetic resonance imaging can depict infection due to the shift of water from local edema, hyperemia and ischemia. The high resolution that can be obtained using this technique facilitates differentiation of bone and soft tissue, and subsequently of osteomyelitis and soft tissue infection [Boutin, 1998, Totty, 1989]. However, artifacts due to ferromagnetic properties of implants can hamper MRI image interpretation [Wegener, 1991]. This is, of course, particularly relevant in diagnosis of prosthesis related infections.

Radioisotopes play an important role in the imaging of infectious and noninfectious disorders in orthopaedics, because radioisotopes or radiolabeled compounds can detect alterations in physiologic activity of tissues. Gamma cameras are used to detect emitted gamma irradiation of intravenously or, for some indications, intraarticularly injected radiopharmaceuticals. Whole body images can be obtained, which might detect a remote focus of arthroplasty infection or can be useful in screening purposes like in fever of unknown origin (FUO). Spot images provide better resolution for depicting focal sites of

infection. Different radiopharmaceutical have been developed that can be used for imaging musculoskeletal infection (Table 1).

In 1971, technetium-(^{99m}Tc)-labeled phosphate complexes were introduced as an agent for skeletal imaging [Subramanian, 1971]. Nowadays, methylene diphosphonate (MDP) or hydroxymethane diphosphonates (HMDP) are used for the bone scan. The mechanism of localization of these complexes is probably driven by the absorption of the phosphonate complex to the bony crystal surface and subsequent slow transport into the bone. Uptake of these radionuclides is partially dependent on blood flow, however increased bone turnover like in fracture or osteomyelitis is thought to be the most important factor in the uptake process [Schneider, 1999]. The radiopharmaceutical is excreted via the urine. Bone scans can be acquired at subsequent time points after intravenous injection of ^{99m}Tc-labeled diphosphonate in a 3- or 4-phase imaging procedure. A series of images obtained in the first phase, one to two minutes after injection gives information about blood flow and vascularity of the lesion. Accumulation of the radionuclide in the extravascular space can be visualized on the blood pool scan in the second phase early (few minutes) after the dynamic flow scan. The third phase of the bone scan includes static images obtained from different directions, 2 to 4 hours after injection, and shows uptake of the ^{99m}Tc-compound by the bone. Eventually, the bone scan can be completed by a fourth stage done 24 hours after injection, which might improve target to background ratio. Three-phase bone scanning with ^{99m}Tc-methylene diphosphonate (MDP) is an excellent tool for the initial

Table 1. Radiopharmaceuticals for scintigraphy in musculoskeletal infection

Vehicle	Radionuclide
Methylene diphosphonate	^{99m}Tc
No vehicle	^{67}Ga-citrate
Leukocytes	^{99m}Tc or ^{111}In
Nonspecific human IgG antibody	^{99m}Tc or ^{111}In
Antigranulocyte monoclonal IgG antibody	^{99m}Tc
Antigranulocyte Fab' antibody fragment	^{99m}Tc
Deoxyglucose	^{18}F
Liposomes	^{99m}Tc
Ciprofloxacin analogue	^{99m}Tc
Antimicrobial peptide	^{99m}Tc

evaluation of bone infection because of its high sensitivity [Elgazzar, 1995]. The three-phase bone scan can be used to differentiate between soft tissue infection and osteomyelitis. Osteomyelitis can manifest itself with increased accumulation due to hyperemia and increased vascular permeability on the first two images, and, in contrast to soft tissue infection, also with delayed uptake on the 2-4 hours image [Wegener, 1991]. The specificity of ^{99m}Tc-MDP to detect osteomyelitis, however, is rather low because high uptake is seen in any area of increased bone turnover. The specificity of ^{99m}Tc-MDP is clearly limited when infection is suspected in previously fractured bone or after surgical intervention [Palestro, 1995]. In addition, the bone scan may be unsuitable for evaluation of installed therapy, as it may remain positive for months after clinical healing has occurred [Scoles, 1980]. Sequential ^{99m}Tc-MDP and gallium-(^{67}Ga)-citrate scintigraphy has been reported to improve specificity in the diagnosis of chronic osteomyelitis [Palestro, 1994, Tumeh, 1986]. Gallium-67 is an analogue of iron that binds to transferrin in blood and extravasates at the site of inflammation [Corstens, 1999, Elgazzar, 1999]. It binds strongly to leukocyte lactoferrins and bacterial siderophores [Tsan, 1985, Wegener, 1991]. After initial excretion by the kidneys, the principle route of excretion after 24 hours is the colon [Palestro, 1994]. ^{67}Ga-citrate - originally developed for bone scanning purposes - can accumulate in bone, which hampers image interpretation in patients with osteomyelitis [Palestro, 1994, Schelstraete, 1992]. Sequential ^{99m}Tc-MDP and ^{67}Ga-citrate scintigraphy has its drawbacks too, especially in patients with sites of increased bone turnover after violation of the bone [Elgazzar, 1995, Palestro, 1995, Sammak, 1999]. ^{67}Ga-citrate imaging with single photon emission computed tomography (SPECT) has recently been reported to offer identical information as that obtained by dual-tracer imaging in patients with spinal osteomyelitis, suggesting that costs and imaging time could be reduced [Love, 2000].

The use of indium-(^{111}In)-labeled leukocytes is nowadays, more than twenty years after it was first reported for clinical application, still considered the gold standard for imaging of infection and inflammation [Corstens, 1999, Thakur, 1977]. However, it requires a relatively cumbersome preparation of labeled autologous leukocytes, which includes the withdrawal and handling of blood. Typically, an image is acquired at 24 hours following injection, which will show normal accumulation in liver, spleen and bone marrow [Sammak, 1999, Wegener, 1991]. Inflammatory or infectious foci attract circulating leukocytes. The role of ^{111}In-leukocytes in the diagnosis of chronic osteomyelitis or prosthesis infection has been debated, because the radiola-

beled leukocytes are mostly polymorphonuclear [Elgazzar, 1999, Oyen, 1991a]. Whereas some authors found only minimal uptake of labeled leukocytes in chronic, low grade infection, others consider the technique to be the method of choice when bone scanning is equivocal [Elgazzar, 1995, Johnson, 1988, Propst-Proctor, 1982, Schauwecker, 1989]. To differentiate normal uptake of labeled leukocytes in marrow from pathologic uptake in infection, combined scintigraphy with labeled leukocytes and marrow tracers like ^{99m}Tc-labeled sulfur-colloid is often necessary [Palestro, 1990, Sammak, 1999]. Unwanted red marrow uptake has also been reported when ^{99m}Tc is used as the radionuclide in leukocyte scintigraphy [Wegener, 1991]. However, ^{99m}Tc linked via hexamethylpropylene amine oxime (HMPAO) to leukocytes, offers several advantages over ^{111}In-leukocytes, such as cost, availability and dosimetry [Corstens, 1999, Sammak, 1999]. A retrospective study on imaging of bone and joint infection in 116 patients suggested that ^{99m}Tc-HMPAO-leukocytes scintigraphy gives results comparable to those obtained with ^{111}In-leukocytes [Devillers, 1995]. In contrast with the *in vitro* radiolabeling of leukocytes, granulocytes can also be radioactively tagged *in vivo*, using a radiolabeled antigranulocyte monoclonal antibody or its Fab fragment. The latter techniques (using technetium-99m as the radionuclide) might improve sensitivity for infection compared to the white blood cell scan [Hakki, 1997, Reuland, 1991]. Rapid imaging within several hours after injection has been reported to be possible with the ^{99m}Tc-labeled antibody fragment (known as LeukoScan), in contrast to whole antibody scintigraphy [Becker, 1999, Becker, 1996]. False-negative imaging of LeukoScan has been reported in patients with chronic osteomyelitis, an involucrum-like formation of a sheath of sclerotic new bone around the infected bone, and leukopenia in an HIV positive patient [Becker, 1996, Hakki, 1997]. Nonspecific, antigen unrelated uptake of antibody due to increase vascular permeability might account for false positive LeukoScan results. This is for instance seen after loosening of a prosthesis, fractured bone and periprosthetic calcification [Becker, 1996, Hakki, 1997]. Again, nonpathological LeukoScan uptake in bone marrow islands might be excluded with sequential ^{99m}Tc-labeled sulfur-colloid scanning [Becker, 1996, Palestro, 1991b].

Positron emission tomography (PET) with fluor-18-deoxyglucose (^{18}FDG) can be used for scintigraphic imaging of cancer, but also for detection of inflammatory and infectious processes. Increased glucose metabolism in inflammatory cells account for the accumulation of ^{18}FDG in the latter. Zhuang *et al*. showed increased accumulation of ^{18}FDG in chronic osteomyelitis on the PET scan, and these authors claimed that this technique was likely to be more

cost-effective than labeled leukocyte scintigraphy for this indication [Zhuang, 2000]. However, other sites of musculoskeletal inflammation like postsurgical inflammatory changes have been shown to cause false-positive results in ^{18}FDG-PET scans for as long as 6 months after the operation.

In view of the problems with existing radiopharmaceuticals, new radiopharmaceuticals have been proposed for imaging of infection such as radiolabeled nonspecific human immunoglobulin (IgG) and radiolabeled liposomes. IgG is a large protein that accumulates in inflammatory tissue by virtue of increased vascular permeability [Rubin, 1994]. Several studies suggest the utility of ^{111}In-IgG scintigraphy for the detection of various types of focal infection, including those located in the bone or joints [Oyen, 1992b, Oyen, 1992d, Rubin, 1989]. Preliminary data suggested that this imaging modality is very useful in the detection of infected arthroplasties [Oyen, 1991b]. Therefore, we evaluated in **Chapter 3** the efficacy of ^{111}In-IgG scintigraphy to detect musculoskeletal infection.

Radiolabeled liposomes (artificial phospholipid vesicles) can also be used for imaging of infection [Boerman, 1995, Goins, 1993, Oyen, 1996a]. Studies suggested that locally enhanced vascular permeability and a local uptake that is driven by high concentration of liposomes, contribute to the mechanism of accumulation of liposomes [Awasthi, 1998, Laverman, 2000, Oyen, 1996b]. Long-circulating characteristics of currently developed liposomes prevent these from rapid clearance by the reticuloendothelial system [Bakker-Woudenberg, 1993, Woodle, 1992]. These so-called sterically stabilized liposomes, coated with polyethyleneglycol (PEG) and radiolabeled with ^{111}In or ^{99m}Tc, showed favorable performance compared to routine agents in various experimental models of acute infection [Boerman, 1995, Oyen, 1996a]. A recent clinical study suggested better visualization of musculoskeletal infection with ^{99m}Tc-labeled PEG liposomes, compared with ^{111}In-IgG [Dams, 2000]. This was attributed to the smaller size of IgG that would result in less retention of the molecule at the site of the infection. A new ^{99m}Tc-labeling method, using hydrazinonicotinamide (HYNIC) as the chelator, was recently shown to improve the *in vivo* and *in vitro* characteristics of radiolabeled PEG-liposomes as compared to the conventional ^{99m}Tc-HMPAO labeling method [Laverman, 1999]. Nonspecific human immunoglobulin (IgG) can also be labeled with ^{99m}Tc, which is to be preferred over the use of the ^{111}In radionuclide, because of better imaging properties and lower radiation dose. Dams *et al.* recently showed that ^{99m}Tc-HYNIC-IgG performed equally well as ^{111}In-IgG in patients with non-acute infection, including some patients with chronic osteomyelitis [Dams, 1998a].

In **Chapter 4**, the performance of ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG to detect chronic osteomyelitis was evaluated in a rabbit model. These agents were compared with ^{99m}Tc-MDP, ^{67}Ga-citrate and ^{111}In-granulocytes.

Technetium-99m-labeled ciprofloxacin derivate (Infecton) and antimicrobial peptides are other examples of newly develop radiopharmaceuticals, which can be used for the imaging of musculoskeletal infection [Vinjamuri, 1996, Welling, 2000]. Supposedly, these substances bind preferentially to bacteria over host cells, thereby distinguishing sterile inflammation from infection.

As molecular insights in medicine are expanding, it is tempting to speculate about the role DNA based techniques in nuclear medicine in the next future. Antisense oligonucleotides have been used for the imaging of cancer [Hildebrandt, 1996, Tavitian, 1998]. In theory, a parallel can be drawn to the imaging of bacterial infections, when such antisense oligonucleotides are directed against specific bacterial gene sequences. Stability, biodistribution and intracellular uptake of oligonucleotide probes might pose major drawbacks for such application [Hildebrandt, 1996].

I Molecular techniques for diagnosis of musculoskeletal infection

Not only the imaging of the infectious process can be met with difficulties; also the detection and identification of the pathogen of musculoskeletal infection can be difficult and/or laborious. Rapid identification, isolation and subsequent determination of antimicrobial susceptibility are particularly important for the tailoring of adequate antimicrobial therapy. However, conventional methods that can identify pathogens of infection have their limitations. Speedy screening methods like Gram staining or acid fast staining characterize microorganisms by their appearance, but their accuracy is low [Krogstad, 1989]. Culture is an important tool for identification of microorganisms and allows for further sensitivity studies. Nevertheless, some shortcomings of culture are recognized that can decrease the chance of successful outcome of all kinds of infection, including musculoskeletal ones [Hoeffel, 1999]. These shortcomings concern the diagnostic delay and sensitivity of culture. Some bacterial species are easily cultured in three days, but especially culture of anaerobic and slow growing organisms like mycobacteria can be

delayed for 2-8 weeks [Krogstad, 1989]. Not infrequently, previous or concurrent antimicrobial treatment in patients with an orthopaedic implant or chronic osteomyelitis disturbs the outcome of microbiology testing. In addition, the capability of some bacterial species to change their phenotype and adapt to the microenvironment on the surface of a foreign body may hamper full recovery of all isolates [Gristina, 1985]. Orthopaedic infections are often associated with foreign bodies or biomaterials, like joint prostheses, osteosynthesis-hardware or allografts [Musher, 1977]. The local release of antibiotics may further enhance the production of so called small colony variants of bacterial strains in polymer associated orthopaedic infections, as shown by von Eiff *et al.* [von Eiff, 1997]. Two decades earlier, Musher *et al.* had demonstrated that variant forms of *S. aureus* emerged after in vitro exposure to gentamicin [Musher, 1977]. Although less virulent than their parent strains, these variant strains were shown to readily induce infection in experimental animals. Growth of small colony variant strains can be considerable reduced, resulting in failure to recognize the bacteria by personnel in the microbiology laboratory [Proctor, 1995, Roggenkamp, 1998, von Eiff, 1998]. Alternatively, other specific growth requirements of microorganisms like anaerobic growth are also difficult to predict. The failure to detect pathogens in revision arthroplasty may be the result of this problem, but may also explain some beneficial effects of antimicrobial therapy in noninfected loosened implants, since it is likely that some of these patients have unrecognized infected implants [Espehaug, 1997, Tunney, 1999].

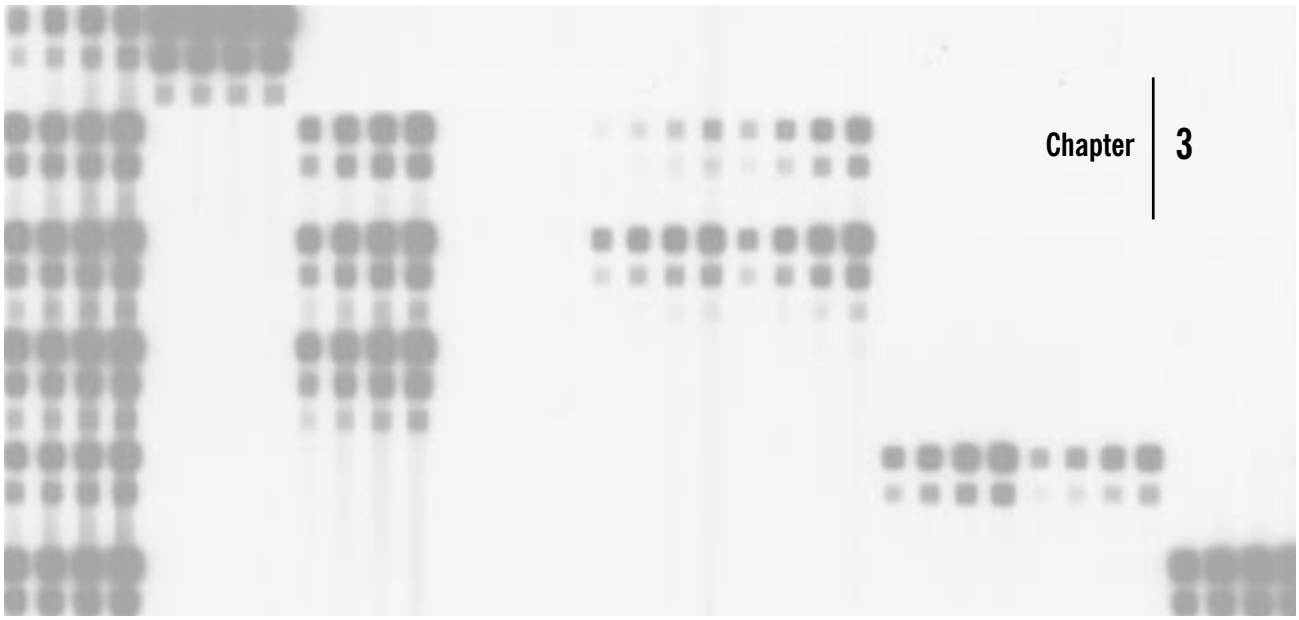
So there is a need for detection techniques, which can bypass these drawbacks in the diagnosis of orthopaedic infections. This might be accomplished with new molecular tools that have been advocated for and are currently under investigation [Garvin, 1995, Hoeffel, 1999, Mariani, 1996, Mariani, 1998, Rantakokko-Jalava, 2000, Tunney, 1999]. These techniques are aimed to detect (amplified) bacterial DNA directly, independently of specific growth characteristics of bacteria. Commonly, most procedures involve polymerase chain reaction (PCR) to amplify DNA. PCR, first described by Mullis *et al.* in 1986, is based on the repetition of a 3-step process: [Loutit, 1995, Mullis, 1986, Mullis, 1987]. (1) denaturing double-stranded DNA into single-stranded DNA by heating the sample; (2) annealing primers (specific synthetic oligonucleotides that determine which part of the gene is amplified) by cooling the sample; and (3) extension of the primers complementary to the single-stranded DNA templates by the DNA polymerase enzyme, thereby doubling the amount of specific DNA present. Repetition of these steps enables PCR to copy one piece of DNA one million times in only 3 hours.

In **Chapter 5**, the development and preliminary clinical results of a new technique for the identification of bacterial PCR products is described. This technique, reverse line blot hybridization (RLB), uses different oligonucleotide probes attached on a membrane to screen for reactivity on clinical specimens after a broad-range PCR on the gene encoding for the 16S-subunit of ribosomal RNA (16S rRNA). Broad-range primers in 16S rRNA PCR are directed against the two ends of a large part (consisting of about 550 base pairs) of the 16S rRNA gene. PCR with these primers will typically result in amplification of only bacterial DNA, since eukaryotic cells do not contain the 16S subunit of rRNA. Within this amplified DNA, several sequences can be identified that are specific to one or more bacterial species. On the internet, large databases are available that contain information on DNA sequences. This information can be used to find the specific sequences, which can be used to design oligonucleotide probes of about 15-20 base pairs in length for identification purposes. Since our aim was to use RLB for the diagnosis of musculoskeletal infections, we designed species-, genus- and group-specific oligonucleotide probes to identify bacteria that are frequently seen in these infections. An additional eubacterial probe screens for other possible bacterial species in the specimen. Accuracy and clinical feasibility of RLB were tested using intraoperative tissue specimens of orthopaedic patients.

I Aims of Part 1 of this thesis

- To evaluate the feasibility of ^{111}In-IgG scintigraphy of musculoskeletal infections in patients (**Chapter 3**);
- To evaluate the performance of ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG for scintigraphy in a rabbit model of chronic osteomyelitis as compared to the agents ^{99m}Tc-MDP, ^{67}Ga-citrate and ^{111}In-leukocytes (**Chapter 4**);
- To develop and evaluate the reverse line blot technique for the diagnosis of orthopaedic infections (**Chapter 5**).

The evaluation of musculoskeletal infections with indium-111-labeled human IgG scintigraphy



Chapter 3

I Introduction

Infection and inflammation of the musculoskeletal system is a common condition in clinical practice. Adequate diagnostic modalities are necessary for optimal planning of treatment. A specific diagnostic challenge is encountered in the group of patients with a painful total joint arthroplasty, since it can be cumbersome to differentiate between two main complications of total joint arthroplasty, i.e. loosening and infection of the implant. Both can present themselves clinically as painful joints, with equivocal or inconclusive follow-up. In the last decades, a wide variety of procedures have been proposed for the diagnosis of either implant-related or other types of musculoskeletal infection. Conventional radiography remains the first-step procedure for this group of patients. Radiographs form the basis of the diagnostic work-up, since bone and joint pathology, other than infection, can be identified at relatively low cost without the need for scintigraphic imaging. However, radiographs alone are seldom diagnostic. Therefore, more sophisticated modalities, such as computerized tomography (CT), magnetic resonance imaging (MRI) and nuclear medicine techniques are used. Focusing on the latter, bone scanning with technetium-99m (^{99m}Tc) diphosphonate is the oldest and most widely used [Wegener, 1991]. Although these agents have superior sensitivity, specificity is low [Elgazzar, 1995, Palestro, 1995, Wegener, 1991]. This is caused by high uptake in areas of increased bone turnover of any cause. This is particularly a problem for differential diagnosis in violated bone, for example after arthroplasty or surgery. Similar problems have been reported for gallium-67 (^{67}Ga) citrate scintigraphy [Elgazzar, 1995, Palestro, 1994]. Labeled leukocyte scintigraphy is currently the most commonly used scintigraphic technique for evaluation of infectious and inflammatory disease, especially when combined with bone marrow seeking tracers, like ^{99m}Tc-labeled sulfur-colloid, in areas where normal bone marrow uptake may be a confusing factor in image interpretation [Palestro, 1990]. However, preparation of labeled leukocytes is relatively cumbersome and requires the handling of patients blood. Moreover, sensitivity in areas of low-grade infection may be decreased [Schauwecker, 1989]. In view of the problems with ^{67}Ga and labeled leukocytes, many new agents are currently being evaluated in clinical practice. One of those agents is indium-111-labeled nonspecific polyclonal human immunoglobulin G (^{111}In-IgG), a labeled large protein that accumulates in inflammatory tissue by virtue of increased vascular permeability [Rubin, 1994]. Several studies suggest the utility of ^{111}In-IgG scintigraphy for the

detection of various types of focal infection, including those located in the bone or joints [Oyen, 1990, Oyen, 1992d, Rubin, 1989]. A preliminary study of Oyen *et al.* showed promising results for the detection of arthroplasty infections [Oyen, 1991b]. In the present study, we evaluated the usefulness of ^{111}In-IgG scintigraphy in routine clinical practice.

I Materials and methods

Patients

The studies of 243 patients, obtained over a period of four years, were reviewed. Of those 243 patients, 226 patients (108 male and 118 female; mean age 54.3 years, with a range of 5-90 years) suspected of 232 possible foci of infection could be evaluated. Previously published patient data, which were obtained before patients were recruited for the present study were not included [Oyen, 1990, Oyen, 1992d]. The patients were categorized on the basis of the suspected focus: total hip arthroplasty (n=87; 38%), total knee arthroplasty (n=17; 7%), chronic osteomyelitis (n=43; 18%), diabetic foot osteomyelitis (n=22; 9%), pseudarthrosis (n=11; 5%), septic arthritis (n=16; 7%), noninfectious arthritis (n=13; 6%), soft-tissue infection (n=7; 3%) or spondylodiscitis (n=16; 7%). Acute osteomyelitis was defined as evidence of infection for one or several days, chronic osteomyelitis as evidence of infection for weeks, months or even years.

Radiopharmaceuticals

DTPA-conjugated human nonspecific polyclonal IgG was obtained as a lyophilized kit containing 2 mg IgG per vial (MacroScint, RW Johnson Pharmaceutical Research Institute, Spring House, PA). The kit was radiolabeled with ^{111}In (indium-111 chloride, Mallinckrodt Medical, Petten, The Netherlands) in a 15-minutes, one-step procedure according to the manufacturer s instructions. The radiochemical purity as determined by instant thin layer chromatography was always higher than 95%. A dose of approximately 2 mg IgG, labeled with 75 MBq ^{111}In was injected intravenously.

Within 1 month of the ^{111}In-IgG scintigraphy, two- or three- phase skeletal scintigraphy was performed in 135 patients after intravenous injection of methylene diphosphonate labeled with 600 MBq ^{99m}Tc. The maximum time interval was only considered in chronic cases and only when no invasive

diagnostic or therapeutic interventions were performed. In acute cases, the studies were all performed within a few days.

Imaging procedure and image interpretation

Scintigraphic imaging was performed as reported earlier [Oyen, 1992d]. In brief, digital images were obtained with a Siemens Orbiter gammacamera connected to a Scintiview image processor (Siemens Inc., Hoffman Estates, IL). Indium-111-IgG images were acquired at 4, 18-24 and 42-48 hours postinjection. All images were interpreted by 3 observers. The observers were not blinded for pretest clinical information. A definite judgment of the scintigraphic images was reached before any of the verification procedures was performed. The ^{111}In-IgG images were interpreted with the corresponding bone scintigraphy, when available. A ^{111}In-IgG scan was interpreted as positive, if focal, continuously increasing accumulation could be noted over time. Nonvisualization of the lesion detected by bone scan and/or plain radiographs, or failure to show increasing accumulation, was considered to be a negative ^{111}In-IgG scintigraphy result. A bone scan was considered positive when there was increased activity in at least two phases (blood pool and late phase) in the area of interest.

Verification

The scintigraphic results were verified by culture, obtained surgically (n=98, 42%) or by puncture (n=43, 19%) or long-term clinical and roentgenological follow-up (n=91, 39%). The latter was mainly used in patients with negative diagnostic work-up, including negative scintigraphic imaging.

I Results

The overall infection prevalence was 27% (62 foci). *Staphylococcus* species were cultured from 17 foci (38%), *Streptococcus* species from 13 foci (29%), *Pseudomonas aeruginosa* from 3 foci (7%) and a variety of other microorganisms or mixed flora from 12 foci (27%).

Nineteen patients received antibiotics before ^{111}In-IgG scintigraphy, including β-lactam antibiotics, (n=17), tetracycline (n=1), and fluorochinolon (n=1). Twelve scintigrams of these patients were scored true-positive, 5 true-negative, one false-positive and one false-negative. Table 1 summarizes the results of ^{111}In-IgG scintigraphy in various patient categories.

Table 1. Results of ^{111}In-IgG scintigraphy for detection of infection in various patients

	n	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
Total hip arthroplasty	87	21	12	54	0	100%	82%	64%	100%
Total knee arthroplasty	17	3	7	7	0	100%	50%	30%	100%
Chronic osteomyelitis	43	15	2	26	0	100%	93%	83%	100%
Diabetic foot infection	22	7	5	10	0	100%	67%	58%	100%
Pseudarthrosis	11	2	8	1	0	100%	11%	20%	100%
Septic arthritis	16	5	2	9	0	100%	82%	71%	100%
Noninfectious arthritis	13	13	0	0	0	100%	—	100%	—
Soft-tissue infection	7	6	0	1	0	100%	100%	100%	100%
Spondylodiscitis	16	2	0	13	1	67%	100%	100%	93%
Overall						99%	77%	67%	99%

TP = true-positive, FP = false-positive, TN = true-negative, FN = false-negative, PPV = positive predictive value, NPV = negative predictive value

Total hip arthroplasty (n=87)

All 21 infected total hip arthroplasties showed increased ^{111}In-IgG uptake on scintigraphy (Figure 1a-b). None of the 54 hip arthroplasties demonstrating normal distribution of ^{111}In-IgG were infected. Most (10 of 11) noninfected cementless hip arthroplasties demonstrating normal ^{111}In-IgG uptake were

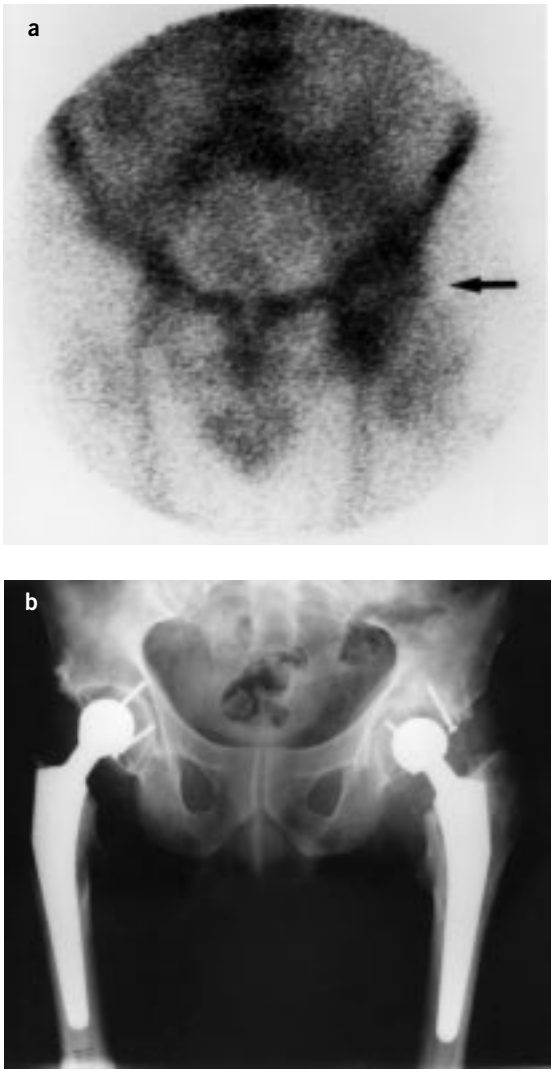


Figure 1. **(a)** ^{111}In-IgG-scintigram of a 69-year-old man showing focal accumulation (arrow) of the radionuclide 15 month after implantation of a left total hip arthroplasty (cemented stem, cementless cup). Intraoperative cultures grew group G β-hemolytic *Streptococcus*. **(b)** Plain radiograph showing a small radiolucent zone in the calcar region and some scalloping around the stem.

implanted 16 months or longer before scintigraphy, whereas cemented hip arthroplasties were recorded as true-negative as early as 6 weeks after implantation. Twelve hip arthroplasties were recorded as false-positive, including 9 cementless implants. Six of the latter had been implanted within a 14-months period before ^{111}In-IgG scintigraphy. In 6 patients with false-positive scintigraphy, histological specimens were available and all showed signs of chronic inflammation near the hip arthroplasty. Other causes of false-positives included foreign body response after wear of the polyethylene socket (n=1), and periarticular ossification (n=1).

One patient with increased ^{111}In-IgG uptake on scintigraphy 6 months after hip arthroplasty still had a wound with signs of inflammation (redness, induration,) at time of imaging. One month later however, this patient was free of complaints. In 4 of 7 hip arthroplasties with increased ^{111}In-IgG uptake located around the neck of the femoral component, no infection was found (Figure 2a-b).

Total knee arthroplasties (n=17)

All three infected knee arthroplasties showed increased ^{111}In-IgG uptake on scintigraphy. Seven of 14 noninfected knee arthroplasties were scored false-positive. In one patient, infection was less likely since 1 month after scintigraphy knee pain decreased and the sedimentation rate of erythrocytes dropped without specific treatment. The positive ^{111}In-IgG scintigraphy in this patient, performed 7 weeks after arthroplasty, remained unexplained, other than being caused by the surgical procedure itself. One false-positive result consisted of increased ^{111}In-IgG uptake on scintigraphy 11 months after total knee arthroplasty, which was caused by calcifications in the knee capsule seen on radiography. Three false-positive results consisted of scintigrams with clear focal ^{111}In-IgG accumulation, but the location of this uptake was noted to be probably caused by noninfectious inflammation of soft tissues rather than infection located in bone around the arthroplasty itself (Figure 3).

Five false-positive results were scored based on negative cultures from needle aspirations (n=4) and arthroscopy (n=1). However, in all four available histological specimens signs of inflammation were seen.

Chronic osteomyelitis (n=43)

All 15 patients with proven chronic osteomyelitis showed increased focal accumulation on ^{111}In-IgG scintigraphy (Figure 4). Two of the 28 foci that were proven to be noninfected were scored as false-positives. The first patient suffered from severe decubitus on the lateral side of her right hip. Soft-tissue

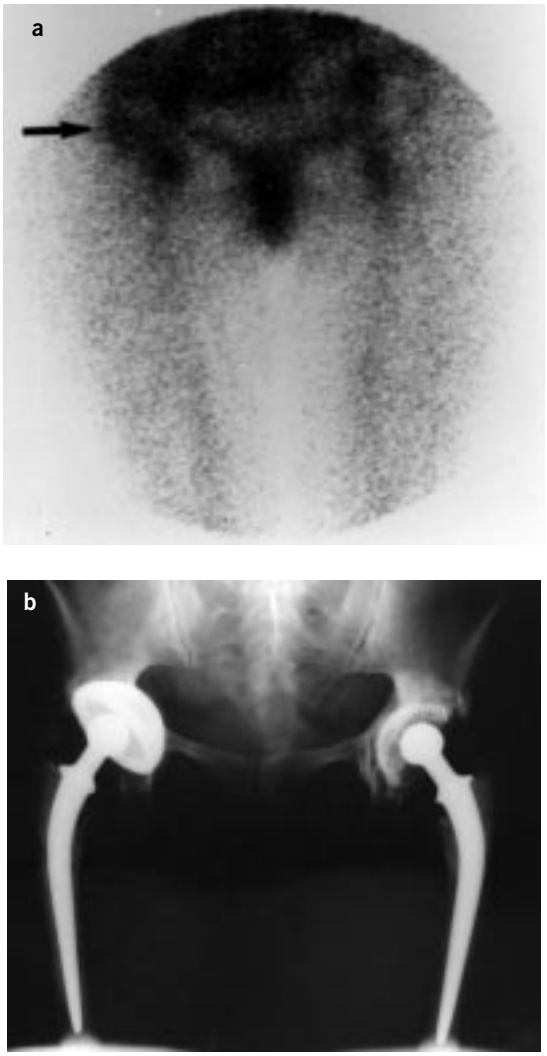


Figure 2. **(a)** ^{111}In-IgG-scintigram of a 46-year-old woman with a cemented right total hip arthroplasty, 2 years after implantation. Cultures obtained intraoperatively and from puncture grew no bacteria. Focal accumulation of ^{111}In-IgG due to aseptic inflammation (histology) is seen around the neck of the femoral component of the arthroplasty (arrow). **(b)** Plain radiograph showing protrusion of the right total hip arthroplasty, a radiolucent zone medial to the stem of the femoral component and periarticular ossifications.

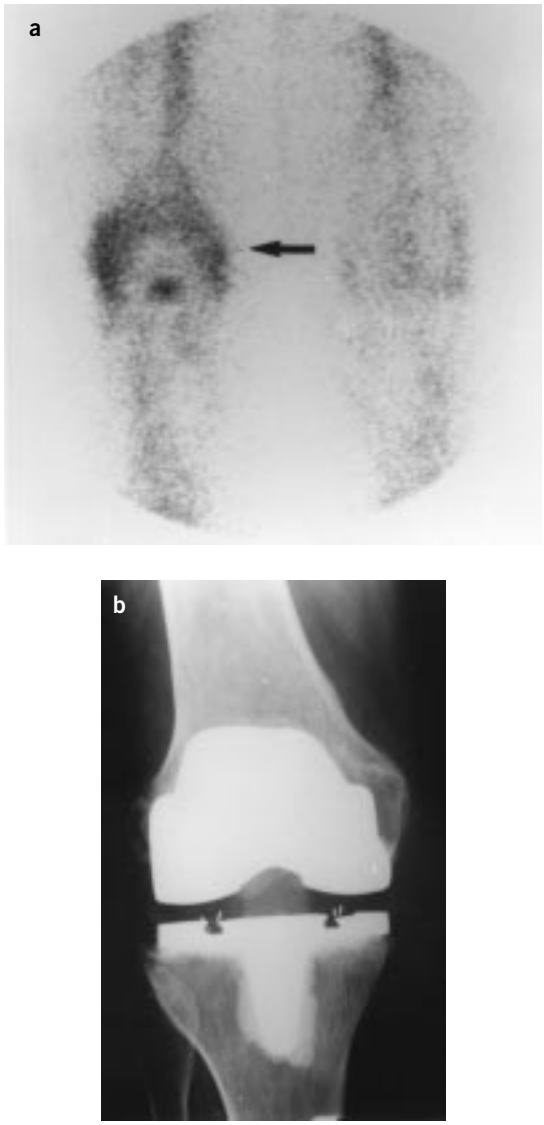


Figure 3. **(a)** ^{111}In-IgG-scintigram of a 71-year-old woman with a painful swollen right knee, 1 year after total knee arthroplasty. Puncture showed no infection. The focal accumulation of ^{111}In-IgG (arrow) was seen to be located in soft-tissues around the arthroplasty and probably caused by aseptic inflammation of soft tissues. Nevertheless the scintigram was interpreted as false-positive for infection. **(b)** Plain radiography showing no radiolucency, only some ossifications in the soft tissues.

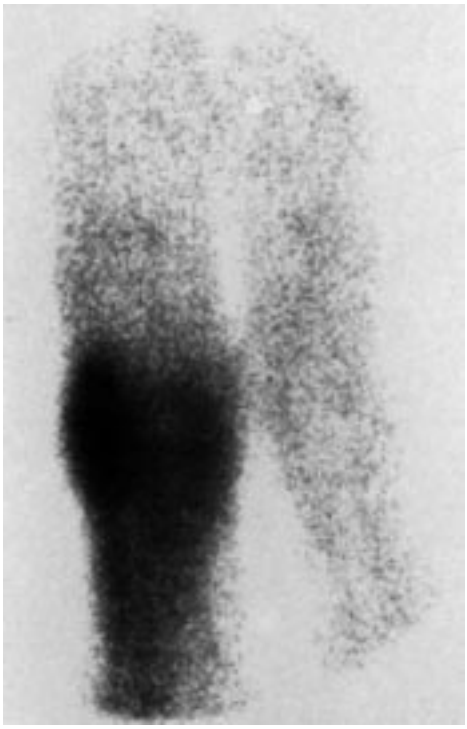


Figure 4. ^{111}In-IgG-scintigram of a 23-year-old man showing focal accumulation in his distal left tibia, which was painful and swollen for 7 years. Intraoperative cultures proved infection (*S. aureus*), and histological specimens showed chronic active osteomyelitis.

cultures grew *Escherichia coli* and *Enterococcus faecalis*. Within 1 month, the wound healed and resection of the head of the hip was not performed. In this case, it was not possible to differentiate between increased ^{111}In-IgG uptake in soft tissue or bone. In the second patient, an above-knee amputation was performed 10 months before scintigraphy due to a late complication of diabetes mellitus.

A fistula at the amputation side existed for several months. Culture of the pus revealed *S. aureus* in low counts. No evident signs of osteomyelitis were seen on plain radiography. Indium-111-IgG scintigraphy was scored as false-positive because the wound healed within 4 weeks after scintigraphy, 3 weeks after oral antibiotics only (amoxicillin-clavulanic acid).

Diabetic foot osteomyelitis (n=22)

Seventeen foci in patients with a diabetic foot were adequately evaluated with ^{111}In-IgG scintigraphy: 7 osteomyelitis, 10 no osteomyelitis (normal scan or soft-tissue inflammation only). Five foci in patients with a diabetic foot were recorded false-positive. Two of those patients had deep ulcers on the heel, culture revealed *S. aureus* and *Proteus vulgaris*. In the first patient histological specimens from the ulcer showed no signs of osteomyelitis, whereas in the other patient the ulcer was healing rapidly 2 weeks after scintigraphy with oral flucloxacillin (4 g/day). Three false-positive results were from patients with Charcot joints with recent fractures, who responded well to conservative treatment.

Pseudarthrosis (n=11)

In only 2 patients, a positive ^{111}In-IgG scintigram could be confirmed as positive for infection. Surgically obtained cultures proved infection in one patient with positive ^{111}In-IgG scintigraphy 3 months after removal of osteosynthesis material from his tibia. In the other patient infection was evident based on a continuing producing fistula for several months from an internal fixator of the femur, being 1 year *in situ* at the time of imaging.

One pseudarthrosis in a tibia with an external fixator placed 16 months before imaging was true-negative, and subsequently proven by a negative culture.

Eight patients with pseudarthrosis showed false-positive focal accumulation of ^{111}In-IgG on scintigraphy. Surgically obtained cultures were negative and no histological evidence for infection was found in these patients. Five out of 8 patients had undergone removal of internal (n=4) or external (n=1) fixators from femur (n=3) or tibia (n=2), at least 3 months before scintigraphy. In the remaining 3 patients the following surgical procedures had been performed: implantation of a dynamic hipscrew one year before imaging, osteotomy of a tibia 5 months and arthrodesis of an ankle 14 months before scintigraphy, respectively.

Arthritis (n=29)

In 13 out of 29 patients the arthritis consisted of noninfectious inflammation in one or more joints. This included gout, severe progressive osteoarthritis, necrosis of the head of the femur, reactive arthritis in response to Crohn s disease and intestinal infection, paraneoplastic polyarthritis and seronegative symmetric polyarthritis. Those joints showed an increased accumulation on ^{111}In-IgG scintigraphy, but with a different distribution-pattern compared to septic arthritis as seen in five other patients. In infectious arthritis, diffuse,

intense uptake is seen in the joint, whereas in sterile arthritis typically the synovial lining is visualized.
In 2 out of 7 patients with ^{111}In-IgG scintigraphy scored positive for infection no septic arthritis could be proven (false-positives). Histological specimens from both joints (knee and ankle) showed mild, chronic synovitis. No signs of infection could be detected in all 9 patients with normal uptake of ^{111}In-IgG in their joints.

Soft-tissue infection (n=7)

The soft-tissue infections consisted of abscess in the psoas muscle region (n=2), abscess in the thigh (n=2), abscess in the calf (n=1) and infection in a Girdlestone-hip (n=2). Six infections were proven by culture and histology and showed increased ^{111}In-IgG uptake on scintigraphy, including 2 patients with AIDS and one patient with leukopenia due to chemotherapy. One Girdlestone-hip with a negative ^{111}In-IgG scintigram was proven to be noninfected by surgically obtained culture.

Spondylodiscitis (n=16)

Spondylodiscitis was proven twice by puncture (lumbar spine), culture revealed *Kingella denitrificans* and *kingae* (Figure 5). Both patients showed focal ^{111}In-IgG accumulation on scintigraphy. In 13 patients with negative ^{111}In-IgG scintigraphy, no spondylodiscitis was detected by long-term follow-up. In one patient, blood cultures revealed *Brucella* species, after which antibiotic therapy was started, 4 days before scintigraphy. Indium-111-IgG scintigraphy showed no increased uptake. MRI showed at this time only early epiduritis, no spondylitis, whereas no signs of infection were seen on CT. Lumbar puncture revealed an acute infection of the central nervous system. Doxycycline (1 dd 200 mg) and rifampicine (1 dd 100 mg) was continued for 4 months. Two and 5 weeks later, MRI showed both epiduritis and spondylitis at L3-L4. Indium-111-IgG scintigraphy was scored as false-negative.

Figure 5. ^{111}In-IgG-scintigram of a 35-year-old man with low back pain. Focal ^{111}In-IgG accumulation is seen on the ventral side of the vertebrae L3-L4. Biopsy proved infection (*K. denitrificans*).

^{99m}Tc-MDP bone scintigraphy

The results of ^{99m}Tc-MDP bone scintigraphy are summarized in Table 2. In all but one patient-category, this imaging technique showed an excellent sensitivity. In the patient with false-negative ^{111}In-IgG scintigraphy for spondylitis, the ^{99m}Tc-MDP bone scintigraphy was also false-negative. Specificity of ^{99m}Tc-MDP bone scintigraphy for osteomyelitis is considerable lower than sensitivity. The ^{99m}Tc-MDP bone scintigraphy remained positive up to 7 months after cemented total hip arthroplasty and 9 months after total knee arthroplasty. All 17 cementless total hip arthroplasties showed increased ^{99m}Tc-MDP on scintigraphy.

Table 2. Results of ^{99m}Tc-MDP bone scintigraphy for detection of infection in various patients

	n	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
Total hip arthroplasty	60	13	40	7	0	100%	15%	25%	100%
Total knee arthroplasty	7	2	4	1	0	100%	20%	33%	100%
Chronic osteomyelitis	26	9	11	6	0	100%	35%	45%	100%
Diabetic foot infection	21	7	13	1	0	100%	7%	35%	100%
Pseudarthrosis	5	1	4	0	0	100%	0%	20%	—
Septic arthritis	9	1	7	1	0	100%	13%	13%	100%
Soft-tissue infection	2	1	0	1	0	100%	100%	100%	100%
Spondylodiscitis	5	2	2	0	1	67%	0%	50%	—

TP = true-positive, FP = false-positive, TN = true-negative, FN = false-negative, PPV = positive predictive value, NPV = negative predictive value

I Discussion

This study shows that ^{111}In-IgG scintigraphy is a very sensitive imaging technique for musculoskeletal infection. In most patient-categories, the sensitivity for infection was optimal, only one patient with spondylitis in the lumbar spine did not show increased ^{111}In-IgG accumulation on scintigraphy. Specificity varies between different patient categories. Specificity is high in patients with total hip arthroplasty, chronic osteomyelitis, arthritis, soft-tissue infection and spondylodiscitis (82%, 93%, 82%, 100% and 100%, respectively), moderate in patients with total knee arthroplasty and diabetic foot pathology (50% and 67%), and low in patients with pseudarthrosis (11%). The high negative predictive value is important for planning, e.g. in prosthesis revision, given the significant extra time and costs of adequate infection treatment. In all foci scored as false-positive with ^{111}In-IgG scintigraphy, signs of sterile inflammation were present. This confirms the present concepts of the mechanism of ^{111}In-IgG accumulation, being not specific for infection, but occurring in any inflammatory focus [Oyen, 1992b, Oyen, 1992d]. In all categories, though, specificity of ^{111}In-IgG scintigraphy is higher compared with ^{99m}Tc-MDP scintigraphy. Indium-111-IgG does not, like ^{99m}Tc-MDP, accumulate in metabolic active bone. Therefore, previous pathologic changes of the bones, like arthroplasty and chronic osteomyelitis, compromise image-interpretation of ^{111}In-IgG scintigraphy less than that of ^{99m}Tc-MDP scintigraphy. An additional advantage of all scintigraphic techniques is that, unlike conventional radiography, CT and MR imaging, altered anatomy itself and metal parts of arthroplasties or osteosynthesis material do not interfere with image interpretation.

Comparing cemented with cementless total hip arthroplasties shows that the period for ^{111}In-IgG scintigraphy to become negative after arthroplasty is considerably longer for cementless total hip arthroplasties. Noninfected cemented hip arthroplasties showed no increase uptake scintigraphy from 6 weeks after implantation, whereas all but one noninfected cementless hip arthroplasties showed false-positive ^{111}In-IgG accumulation on scintigraphy when recorded within a 14 months period after arthroplasty. Therefore, differentiation between cemented and cementless total hip arthroplasty is important for image interpretation of ^{111}In-IgG scintigraphy in the first year after arthroplasty. Prolonged focal uptake of radiolabels in noninfected cementless hip arthroplasties has also been shown in other scintigraphic image modalities. Oswald *et al*. showed increased ^{99m}Tc-MDP and ^{111}In-labeled leukocytes

uptake up to 2 years after cementless hip arthroplasty [Oswald, 1989, Oswald, 1990]. In our study, all cementless hip arthroplasties showed focal accumulation of ^{99m}Tc-MDP on scintigraphy. Wegener and Alavi hypothesized that ingrowth of bone and fibrous tissue in the porous coating of the cementless prosthesis caused this prolonged uptake of the tracer on scintigraphy [Wegener, 1991]. The finding of nonpathologic increased ^{99m}Tc-MDP uptake on bone scintigrams of cemented arthroplasties up to 7 months or even longer after implantation confirms data in literature [Utz, 1986]. In general, aseptic loosening of an arthroplasty caused increased uptake of ^{99m}Tc-MDP, but not of ^{111}In-IgG, indicating the usefulness of the latter technique in differential diagnosis. The main benefit of bone scintigraphy in patients with arthroplasties is to exclude infection [Elgazzar, 1995]. However, the present study indicates that this is relevant in only a minority of patients, in whom no increased accumulation of ^{99m}Tc-MDP is observed. Nevertheless, this imaging technique remains helpful in evaluating infected arthroplasties, because it is widely available, relatively inexpensive and useful to detect aseptic loosening and to localize osseous structures, thus facilitating interpretation of ^{111}In-IgG scintigraphy. Gallium-67 scintigraphy lacks specificity in patients with arthroplasties as well, since gallium-67 can accumulate in areas of noninfected increased bone turnover [Elgazzar, 1995]. Combined bone and ^{67}Ga-scintigraphy increases the specificity of either technique, resulting in an accuracy between 60% and 80% [Palestro, 1995]. To increase specificity of labeled-leukocyte scintigraphy, combined imaging with ^{99m}Tc-labeled sulfur-colloid has been proposed [Palestro, 1990]. This combined technique can differentiate between normal and pathologic uptake of labeled leukocytes in marrow. Reported specificity for this dual-tracer technique ranges between 85% and 100% [Palestro, 1990]. However, dual tracer imaging is less efficient than single tracer imaging, like ^{111}In-IgG scintigraphy [Love, 2000, Palestro, 1995].

Periarticular ossification and debris formed by polyethylene socket wear, both clearly visualized on plain radiography, are other causes of sterile inflammation after total hip arthroplasty [Oyen, 1992d]. However, in most cases, image interpretation does not need to be impaired when the ^{111}In-IgG images are read in conjunction with radiographs. Despite our impression that non-bone-marrow uptake of ^{111}In-IgG around the neck of the femoral component of a hip arthroplasty (outside the osseous structures) could be caused by such noninfectious reaction after socket wear, the scintigraphy was scored positive, since uptake was clearly elevated. Only 3 of 7 total hip arthroplasties proved to be infected, when this pattern of uptake was seen.

Due to the high prevalence of noninfectious inflammation after total knee

arthroplasty, the specificity of ^{111}In-IgG scintigraphy is relatively low in this patient category. It seems possible to increase this specificity to 71% by differentiating between increased ^{111}In-IgG uptake around the knee arthroplasty due to noninfectious inflammation of soft tissues (horseshoe shaped, mild uptake in the suprapatellar bursa above the prosthesis) and uptake due to infection of the knee arthroplasty itself (diffuse uptake, also located in bone). Persistent periprosthetic ^{99m}Tc-MDP uptake for several years like in total hip arthroplasties limits the role of this scintigraphic technique in total knee arthroplasties even more [Rosenthall, 1987]. Little has been reported on other scintigraphic imaging techniques of knee arthroplasty. One study reported a 95% accuracy of combined leukocytes and sulfur colloid imaging in these patients [Palestro, 1991b].

These results are in accordance with a previous study, which showed that ^{111}In-IgG scintigraphy is accurate for evaluating chronic osteomyelitis [Oyen, 1992d]. Controversy exists about the usefulness of leukocyte (neutrophil)-labeled scintigraphy in diagnosing chronic osteomyelitis, with predominant infiltration of mononuclear cells [Elgazzar, 1995, Datz, 1994]. As stated above, previous pathologic changes of bone, like chronic osteomyelitis, do not reduce specificity of ^{111}In-IgG scintigraphy. Not surprisingly, specificity of ^{99m}Tc-MDP scintigraphy is considerably lower in this group of patients (55% versus 93%).

It was difficult to distinguish infection in soft tissue from osteomyelitis with ^{111}In-IgG scintigraphy, particularly in diabetic foot patients with deep ulcers on the heel and in patients with decubitus and a deep fistula . Although bone scanning is essential for localizing osseous structures, separating bone from soft-tissue infection was not possible in these patients. As indicated by Ezuddin *et al.*, SPECT imaging may be helpful to differentiate these two conditions [Ezuddin, 1992]. The ability of MRI to separate bone from soft-tissue infection is a major advantage of this technique for diagnosing osteomyelitis in diabetic foot patients. Yuh *et al.*, in a study of 24 diabetic foot patients, found a 100%-specificity of MRI for osteomyelitis [Yuh, 1989]. Indium-111-labeled leukocyte studies as well reported good results in this patient category, although sensitivity and specificity varied from 79% to 100% and from 68% to 95% respectively [Keenan, 1989, Kolindou, 1996, Larcos, 1991]. A Charcot joint with a recent fracture can be the cause of a false-positive result of ^{111}In-IgG scintigraphy, as was reported in an earlier study of 16 diabetic foot patients [Oyen, 1992c].

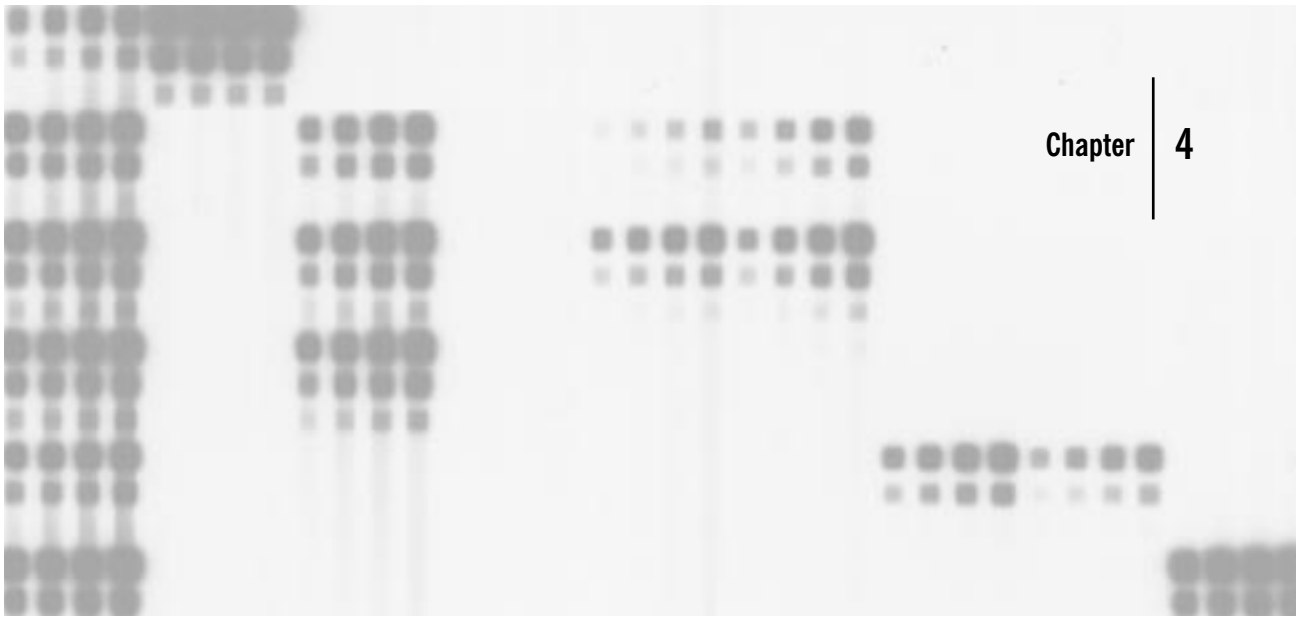
Specificity of ^{111}In-IgG scintigraphy for infection in pseudarthrosis is very low (11%). Nepola *et al.* reported an 82%-specificity (59/70) of combined

labeled leukocyte and bone scintigraphy for infections of delayed unions or nonunions [Nepola, 1993]. Their explanation for the false-positive results in unstable fractures was that these were caused by continued irritation from motion at the unstable sites. This is probably true as well for the nonspecific ^{111}In-IgG uptake on scintigraphy in pseudarthrosis.

Indium-111-IgG scintigraphy visualizes septic arthritis as well as noninfectious inflammatory pathology of the joints, such as metabolic, reactive and autoimmune arthritis. As indicated above, in most patients, the pattern of ^{111}In-IgG uptake on scintigraphy is different for septic and noninfectious inflammatory arthritis. Detailed clinical information is helpful for a correct image interpretation of ^{111}In-IgG scintigraphy of these patients. An advantage of the use of this and other radiopharmaceuticals in the detection of (poly-)arthritis activity is that it allows direct imaging of joints by means of whole-body scintigraphy and of joints that are difficult to assess clinically or radiographically [de Bois, 1995]. The true-positive-scored ^{111}In-IgG scintigraphy in this study of three immunocompromized patients (AIDS and leukopenia due to chemotherapy) with soft-tissue infections confirm that it is possible to detect infections in these patients with this imaging-technique [Oyen, 1992a, Rubin, 1994]. Because these patients are very susceptible for infection and infection often presents atypically in these patients, reliable diagnostic techniques are needed. Some disagreement exists over whether labeled leukocyte scintigraphy is sensitive enough for this purpose [Datz, 1991, Datz, 1994]. The only case of bone infection missed by ^{111}In-IgG scintigraphy in present study concerned a patient with early, low-grade spondylodiscitis. Lower sensitivity for osteomyelitis of the spine has been reported in studies using other scintigraphic techniques as well, including labeled leukocyte and ^{99m}Tc-IgG scintigraphy [Goh, 1990, Palestro, 1991a, Sciuk, 1991, Whalen, 1991]. Modic *et al*. reported a 96% sensitivity using MRI to detect vertebral osteomyelitis [Modic, 1985]. MRI is more useful than CT scanning in this group of patients because it provides better anatomic detail and can differentiate between vertebral and soft-tissue infections [Totty, 1989, Unger, 1988].

In conclusion, ^{111}In-IgG imaging is a sensitive technique for evaluation of infections and inflammatory bone and joint disease. Specificity is increased over ^{99m}Tc-MDP bone scintigraphy, which in most cases is the first procedure performed on these patients except for those with recently violated bone. Specificity of ^{111}In-IgG scintigraphy for infection can be increased even more when considering particular patterns of increased uptake that are frequently caused by sterile inflammatory processes (e.g., uptake around the neck of a total hip arthroplasty or uptake in the synovial lining only).

Scintigraphic evaluation of experimental chronic osteomyelitis



Chapter 4

I Introduction

Chronic osteomyelitis is a disabling disease which may have a substantial impact on the quality of life [Lew, 1997]. Accurate assessment of the severity and extent of the disease is essential to facilitate and optimize surgical and/or antibiotic treatment. For this purpose, different diagnostic modalities are available, including conventional radiographs, CT, MRI and scintigraphic techniques. Three-phase bone scanning with ^{99m}Tc-methylene diphosphonate (MDP) is an excellent tool for the initial evaluation of bone infection because of its high sensitivity [Elgazzar, 1995]. Its specificity, however, is rather low because the agent accumulates in any area of increased bone turnover. This is particularly a problem when an additional pathological condition is present, e.g., a fracture or an orthopedic device. In addition, the bone scan may be unsuitable for evaluation of installed therapy, as it may remain positive for months after clinical healing has occured [Scoles, 1980]. Sequential ^{99m}Tc-MDP and ^{67}Ga-citrate scintigraphy has been reported to improve specificity in the diagnosis of chronic osteomyelitis [Palestro, 1994, Tumeh, 1986]. Unfortunately, ^{67}Ga-citrate also accumulates at sites of increased bone turnover, hampering correct interpretation in patients with violated bone. The role of ^{111}In-leukocytes in the diagnosis of chronic osteomyelitis is still controversial. Although some authors found only minimal uptake of labeled leukocytes in chronic, low grade infection, others consider the technique to be the method of choice when bone scanning is equivocal [Elgazzar, 1995, Johnson, 1988, Propst-Proctor, 1982]. Major disadvantages of radiolabeled leukocytes are the laborious procedure and the need to handle potentially contaminated blood.

New radiopharmaceuticals proposed for imaging of infection are radiolabeled nonspecific human immunoglobulin and radiolabeled liposomes. ^{111}In-immunoglobulin G (IgG) has been extensively studied in patients with musculoskeletal infections, and the agent has shown high accuracy in chronic osteomyelitis [Oyen, 1992d]. Recently, it has been showed that IgG labeled to ^{99m}Tc via hydrazino nicotinamide (^{99m}Tc-HYNIC-IgG) performed equally well as ^{111}In-IgG in patients with non-acute infection, including some patients with chronic osteomyelitis [Dams, 1998a]. The application of radiolabeled liposomes for imaging purposes has regained interest with the development of liposomes with long-circulating characteristics [Bakker-Woudenberg, 1993, Woodle, 1992]. These so-called sterically stabilized liposomes, coated with polyethyleneglycol (PEG) and radiolabeled with ^{111}In or ^{99m}Tc, showed

favorable performance compared to routine agents in various experimental models of acute infection [Boerman, 1995, Oyen, 1996a]. A new ^{99m}Tc labeling method, using HYNIC as the chelator, was recently introduced and showed improved *in vivo* and *in vitro* characteristics of radiolabeled PEG-liposomes compared with the conventional ^{99m}Tc-hexamethyl propylene oxime labeling method [Laverman, 1999]. However, the performance of radiolabeled liposomes has not been evaluated in a model of chronic infection. As a ^{99m}Tc-label is preferred over ^{111}In, on account of better imaging properties and lower radiation dose, we evaluated ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG in a rabbit model of chronic osteomyelitis. For comparison, we included the agents ^{99m}Tc-MDP, ^{67}Ga-citrate and ^{111}In-granulocytes.

I Materials and methods

Animal model

Adult female New Zealand White rabbits ranging in weight from 2.8 to 3.2 kg were caged individually, and fed with regular rabbit diet and water *ad libitum*. The experiments described in this paper were carried out in accordance with the guidelines of the local animal welfare committee. Chronic osteomyelitis was induced in nine rabbits as described previously with minor modifications [Nijhof, 1999]. Briefly, the rabbits were anesthetized with a mixture of halothane, nitrous oxide and oxygen, and placed prone on the operation table. Both hind legs were shaved, disinfected with a 2% tincture of iodine and isolated by sterile drapes. The trochanter tertius was exposed bilaterally and the cortex was penetrated using an air-pressured AO minidrill. The hole was widened and the femoral canal was reamed with drills and fraises. The medullary canal was washed with sterile saline solution and suctioned. A small syringe with a 2-cm long silicone tube (outer diameter 3.0 mm) attached to it was filled with bone cement (Simplex P; Stryker-Howmedica-Osteonics, Rutherford, NJ), placed in an applicator gun and approximately 1.2 ml cement was injected gently into the right femoral canal. The left femoral canal was then inoculated with 10^6 CFU *Staphylococcus aureus*, and closed with bone cement as described above. After polymerization of the cement, the wounds were cleaned with sterile saline solution and closed. Clinical examination was performed regularly with special attention to wound healing, activity level and body temperature and body weight.

Radiopharmaceuticals

^{99m}Tc-PEG-HYNIC-liposomes. PEG-HYNIC-liposomes were prepared as described previously [Laverman, 1999]. The liposomes were composed of the polyethyleneglycol-2000 derivative of distearoylphosphatidyl-ethanolamine (PEG-DSPE), partially hydrogenated egg-phosphatidylcholine, cholesterol and the hydrazino-nicotinamide derivative of distearoylphosphatidyl-ethalonamine (HYNIC-DSPE) in a molar ratio of 0.15:1.85:1:0.07. The particle size distribution was determined by dynamic light scattering with a Malvern 2000 system equipped with a 25-mW Neon laser (Malvern Instruments Ltd, Malvern, United Kingdom). As a measure of particle size distribution of the dispersion, the polydispersity index was determined. This index ranges from 0.0 for an entirely monodisperse dispersion to 1.0 for a completely polydisperse dispersion. The mean size of the liposome preparations was 85 nm with a polydispersity index of 0.1. Preformed HYNIC-PEG liposomes were labeled with ^{99m}Tc as previously described [Laverman, 1999]. ^{99m}Tc-labeded HYNIC liposomes have been shown to be highly stable. No significant release of radiolabel was observed after incubation with high concentrations of diethylenetriamine pentaacetic acid, cystine, or glutathione or after 48 hours of incubation in serum at 37¡C [Laverman, 1999]. The radiochemical purity of the PEG liposomes was determined using instant thin-layer chromatography (ITLC) on ITLC-SG strips (Gelman Sciences, Inc., Ann Arbor, MI) with 0.15 mol/l sodium citrate (pH, 5.0) as the mobile phase and verified by elution on a PD-10 column. Labeling efficiency exceeded 95%, and the ^{99m}Tc-liposomes were administered without any further purification (37 MBq per rabbit).

^{99m}Tc-HYNIC-IgG. HYNIC was synthesized and conjugated to human polyclonal IgG (Gammagard; Baxter/Hyland, Lessines, Belgium) according to the method described by Abrams *et al* [Abrams, 1990]. The purified HYNIC-conjugated IgG was diluted to 4 mg/ml in 0.15 Mol/l acetate (pH, 5.85), sterilized by membrane filtration and stored at -20¡C in 0.5-ml aliquots. After thawing of 0.5 ml of the HYNIC-IgG-conjugate, the conjugate was radiolabeled with ^{99m}Tc by adding 0.1 mg N-[Tris(hydroxymethyl)methyl]glycine (Fluka, Buchs, Switzerland), 0.01 mg $SnSO_4$ and 400 MBq ^{99m}Tc pertechnetate. The mixture was incubated for 15 minutes at room temperature. The radiochemical purity of the radiolabeled IgG was determined by ITLC on silica gel strips with 0.15 mol/l sodium acetate (pH, 5.85) as the mobile phase. High-performance liquid chromatography analysis of the ^{99m}Tc-HYNIC-IgG preparation on a size-exclusion column (Protein Pak 300 SW; Waters Associates, Milford, MA) revealed that the preparation migrated as a monomeric 150-kDa peak (<5% aggregrates) as has been described

[Claessens, 1996]. Labeling efficiency was always >95%. Each rabbit received a dose of 0.2 mg IgG labeled with approximately 37 MBq ^{99m}Tc.

^{67}Ga-citrate. ^{67}Ga-citrate (DRN 3103) was purchased from Mallinckrodt, Inc. (Petten, The Netherlands). A dose of approximately 18 MBq ^{67}Ga-citrate per rabbit was injected intravenously.

^{111m}In-granulocytes. Carotid artery canulation was performed on 2 anesthetized donor rabbits. A total of 100 ml of blood was drawn into acid citrate dextrose-coated tubes. The total leukocyte count of the donor rabbits was 6.4 and 6.8x10^9/l, respectively, with approximately 50% granulocytes. Separation of granulocytes was performed according to the method described by Lillevang *et al.* with minor modifications [Dams, 1998b, Lillevang, 1994]. As we have shown previously, this separation procedure did not affect granulocyte function [Dams, 1998b]. Morphological integrity of the granulocytes was checked by light microscopy. Granulocyte purity (Giemsa-stained slides) was >90%. Functional integrity of the labeled granulocytes was checked by trypan blue staining, indicating that cell viability exceeded 98%. In addition, granulocyte function was evaluated by *in vivo* performance, including transit through the lungs and recovery of labeled granulocytes in the blood. The labeling efficiency was 86%. A dose of 18 MBq ^{111}In-granulocytes was administered intravenously.

^{99m}Tc-MDP. A kit containing methylenediphosphonate and stannous chloride was labeled with ^{99m}Tc, with a labeling efficiency > 95% as determined by ITLC. A dose of 18 MBq ^{99m}Tc-MDP was administered intravenously.

Study design

All rabbits underwent studies with ^{99m}Tc-liposomes, ^{99m}Tc-IgG and ^{99m}Tc-MDP to minimize bias caused by variation in the degree of infection. The relatively long half-lives of ^{111}In and ^{67}Ga precluded serial injection. Therefore, the animals were then randomized into two groups to receive either ^{67}Ga-citrate or ^{111}In-leukocytes. Scintigraphic studies were started 4 weeks after surgery. The radiopharmaceuticals were injected in a fixed order. On day 1 of the imaging experiment, ^{99m}Tc-liposomes were injected. ^{99m}Tc-IgG was administered on day 2, followed one day later by ^{99m}Tc-MDP. On day 4, one group of rabbits received ^{111}In-granulocytes and the other group ^{67}Ga-citrate. The rabbits were slightly sedated by a subcutaneous injection of 0.2 ml Hypnorm (fentanyl, 0.315 mg/ml and fluanisone, 10 mg/ml; Janssen Pharmaceutical, Oxford, UK). After sedation, the rabbits were immobilized in a mold and placed prone on a gamma camera equiped with a parallel-hole, low-energy collimator (Orbiter, Siemens Medical Systems Inc., Hoffman Estate, IL) for

the ^{99m}Tc studies and a medium-energy collimator for the ^{67}Ga and ^{111}In studies. Imaging was performed at 5 minutes, 1, 4, 10, 22 and 44 hours after injection of ^{111}In or ^{67}Ga. Imaging with ^{99m}Tc-MDP was performed at 3 minutes after injection (blood-pool image), and 1 and 4 hours after injection (delayed images). Images (250,000 counts/image, except the 22-h postinjection image [100,000 counts/image]) were obtained and stored in a 256 x 256 matrix.

The scintigraphic results were analyzed quantitatively and qualitatively. Regions of interest (ROI) were drawn over the infected right femur and sham-operated left femur, and over the whole body. Additional ROIs over the lungs were drawn on the scintigrams of rabbits injected with ^{111}In-labeled granulocytes. Ratios of infected right femur to sham-operated left femur were calculated. Residual activity at the osteomyelitis site compared to whole-body activity was also calculated. Counts were corrected for differences in the numbers of pixels before calculating ratios and percentages. All scans were also evaluated qualitatively without knowledge of the histopathological outcome. Scan findings were considered positive if focal accumulation of radioactivity at the osteomyelitis site exceeded the uptake at the sham-operated site, or if inhomogeneously enhanced accumulation was present at the osteomyelitis site.

A separate group of 2 noninfected rabbits was injected with ^{111}In-granulocytes to calculate granulocyte recovery. Serial blood samples were taken at 5, 15, 30 and 45 minutes and 1, 2, 4 and 22 hours after injection.

Assessment

The results of the scintigraphic studies were compared to the results of radiological, microbiological and histopathological examination. Conventional radiographs were obtained immediately after surgery and before additional imaging was performed. Unaware of the results of all other procedures, observers evaluated the radiograms with respect to 3 parameters: periostal reaction, new bone formation, and extent of bone destruction [Norden, 1980]. Immediately after completion of the scintigraphic studies, the rabbits were killed with an overdose of sodium phenobarbital. The left and right femur from all animals were excised and cleaned of tissue debris. The distal ends were removed and the femur was cut longitudinally in two halves using a high-speed dental drill with a circular metal saw. The bone cement was carefully removed. The external surface of the bone specimens was thoroughly cleaned with alchohol. One bone specimen of each femur was sent for microbiological examination. The other bone specimens were fixed in 4%

buffered formalin and decalcified in 10% ethylenediamine tetraacetic acid. Longitudinal sections were made, slide-mounted, and stained with hematoxylin and eosin. All sections were reviewed with light microscopy with respect to 4 parameters: necrotic bone, purulent inflammation, periostal new bone, and granulation tissue. The presence of osteomyelitis was confirmed on the basis of these histopathological findings.

Statistical analysis

All mean values are given as percentage injected dose per gram (%ID/g) or ratios – one standard error of the mean (SEM). One-way ANOVA was used to compare the uptake at the osteomyelitis site and the sham-surgery site for the different agents. In addition, the repeated-measures ANOVA model was used to evaluate differences among the imaging times for each agent. The level of significance was set at $p < 0.05$.

I Results

One of the 9 rabbits died of a Klebsiella spp. sepsis 2 days after surgery. A second rabbit died of unknown causes after performing ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG scintigraphy. In this rabbit, both studies were positive for infection, confirmed by histopathological analyzis. The following section refers to the data of the 7 rabbits that completed all studies.

Postoperative radiograms showed no fractures or cement outside the femur. Of the 7 rabbits, 6 were found to have histopathological evidence of chronic osteomyelitis in the right femur. In 4 of these rabbits, the infection was confined to the distal femur, whereas in 2 rabbits the infection extended towards the proximal femur. The right limb of the osteomyelitis-negative rabbit showed only minimal leukocyte infiltration, which was found to be consistent with a foreign body reaction. None of the sham-operated left limbs showed any sign of infection. The microbiological studies of the bone specimens were concordant with the histopathological findings in 5 of the 6 infected rabbits, and confirmed the presence of *S. aureus* infection. In contrast, radiological findings were abnormal in only two of these rabbits, showing periosteal elevation and new bone formation at the site of the infection. Scintigraphic analysis was performed on the images of all 7 rabbits. For comparison of the absolute and relative uptake of the respective agents at the osteomyelitis site,

only the data of the six infected rabbits were used. A summary of the results is given in Table 1.

Examples of scintigraphic recordings of the 5 radiopharmaceuticals are shown in Figure 1. Scintigraphy with ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG visualized the infected femur in all 6 osteomyelitis-positive rabbits. These scintigrams were correctly classified as positive for osteomyelitis. In contrast, scintigraphy with both ^{67}Ga-citrate and ^{111}In-granulocytes gave equivocal results each in one infected rabbit. Both rabbits had moderate

Table 1. Results of scintigraphic, radiological, microbiological and histopathological procedures in rabbits with chronic osteomyelitis.

Rabbit	^{99m}Tc-MDP	Liposomes	IgG ^{111}In-granulocytes		^{67}Ga citrate	Radiology	Histology	Culture
	4 hr p.i.	22 hr p.i.	22 h p.i.	44 h p.i.	44 h p.i.			
A	+	+	+	+	N.P.	+	+	+
B	+	+	+	N.P.	+	+	+	+
C	+	+	+	N.P.	+	-	+	+
D	+	+	+	+	N.P.	-	+	+
E	+	+	+	N.P.	±	-	+	+
F	-	+	+	±	N.P.	-	+	-
G	±	+	+	±	N.P.	-	-	-

p.i. = after injection ; liposomes = ^{99m}Tc-PEG liposomes; IgG = ^{99m}Tc-HYNIC-IgG; + = positive; N.P. = not performed; - = negative; ± = equivocal.

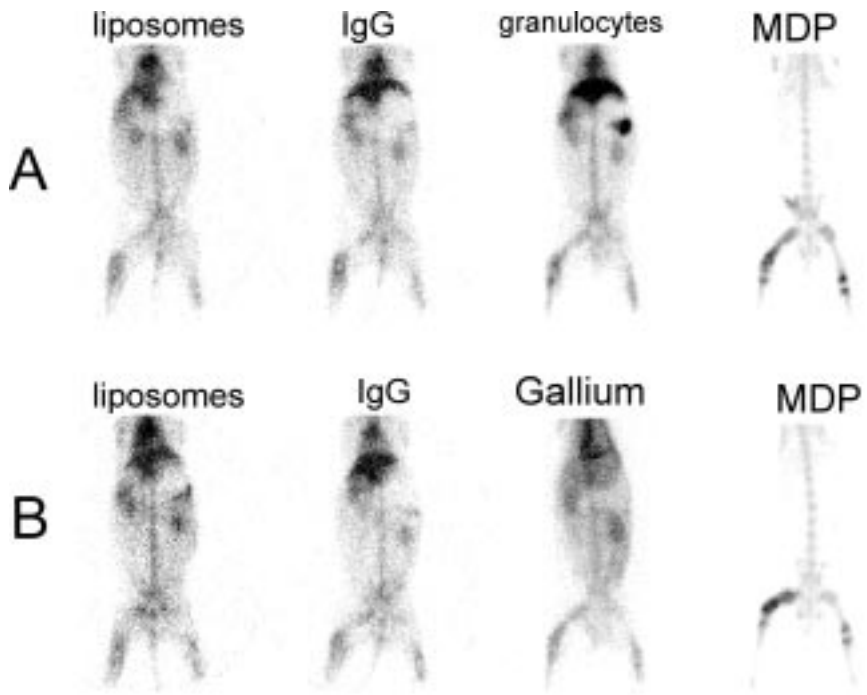


Figure 1. Scintigraphic images of 2 rabbits with chronic osteomyelitis after injection of ^{99m}Tc-MDP (4 h after injection), ^{99m}Tc-PEG-liposomes (22 h after injection), ^{99m}Tc-HYNIC-IgG (22 h after injection), ^{111}In-granulocytes (44 h after injection, rabbit A), and ^{67}Ga-citrate (44 h after injection, rabbit B). Focal uptake of the respective agents in the infected femur is clearly visible.

infection confined to the distal femur. ^{99m}Tc-MDP findings were falsely negative in one of these rabbits. A false-positive result was noted with ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG in the osteomyelitis-negative rabbit. In this case, the scintigrams of ^{99m}Tc-MDP and ^{111}In-granulocytes were classified as equivocal (^{67}Ga-citrate was not performed). The cellular infiltration that was seen on histopathological examination, albeit minimal, probably explained the false-positive result. In 5 of the 6 infected rabbits, scintigraphy with ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG already gave positive results at 4 hours after injection. At 22 hours postinjection, however, visualization of the infection was improved as a result of increasing focal uptake and decreasing background activity. With ^{67}Ga-citrate and ^{111}In-

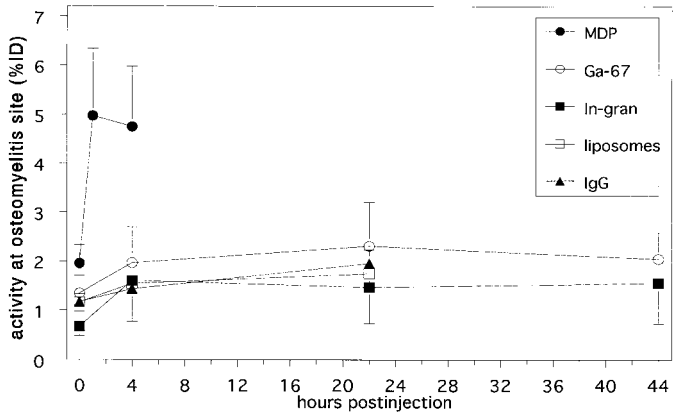


Figure 2. Activity uptake at the osteomyelitis site as determined by quantitative analysis of scintigraphic images of rabbits injected with ^{99m}Tc-MDP (●), ^{99m}Tc-PEG-liposomes (□), ^{99m}Tc-HYNIC-IgG (▲), ^{111}In-granulocytes (■), and ^{67}Ga-citrate (◯), comparing uptake at the osteomyelitis site. Error bars represent SEM.

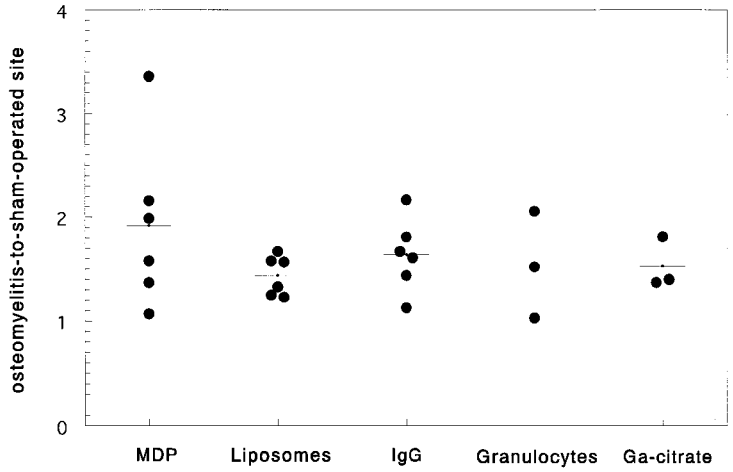


Figure 3. Ratios of osteomyelitis-to-sham-operated site at 4 h (^{99m}Tc-MDP), 22 h (^{99m}Tc-PEG-liposomes, ^{99m}Tc-HYNIC-IgG) and 44 h (^{111}In-granulocytes, ^{67}Ga-citrate) after injection, calculated from quantitative ROI analyses of the scintigraphic images. Horizontal bars represent mean values.

granulocytes scintigraphy, the osteomyelitis could be identified first on the 22-h images. However, with both agents visualization of the infected femur was better on the 44-h images, as a result of improved background clearance. Results of the quantitative analysis of the images are shown in Figure 2. Although ^{99m}Tc-MDP tended to have higher values for uptake in the infected femur, the differences with the other agents were statistically not significant. The residual activity at the osteomyelitis site as a fraction of the whole-body activity, however, was significantly higher than that of the other four agents because of the rapid whole-body clearance of ^{99m}Tc-MDP ($p<0.01$; data not shown). ^{99m}Tc-HYNIC-PEG-liposomes, ^{99m}Tc-HYNIC-IgG, ^{67}Ga-citrate and ^{111}In-granulocytes displayed very similar absolute uptake values at all time points ranging from 1.6 to 2.0 %ID/g in the final image (Figure 2). As shown in Figure 3, ratios of infected-to-sham-operated site were similar for all five radiopharmaceuticals. In accordance with the qualitative assessment of infection, the ratios for both ^{99m}Tc-HYNIC-PEG-liposomes and ^{99m}Tc-HYNIC-IgG increased over time up to 22 hours after injection (1.31 – 0.09 and 1.36 – 0.13, respectively, at 4 hours after injection, and 1.44 – 0.08 and 1.64 – 0.14, respectively, at 22 hours after injection; $p < 0.05$). The difference between the mean ratios of ^{67}Ga-citrate and ^{111}In-granulocytes in the 22- and

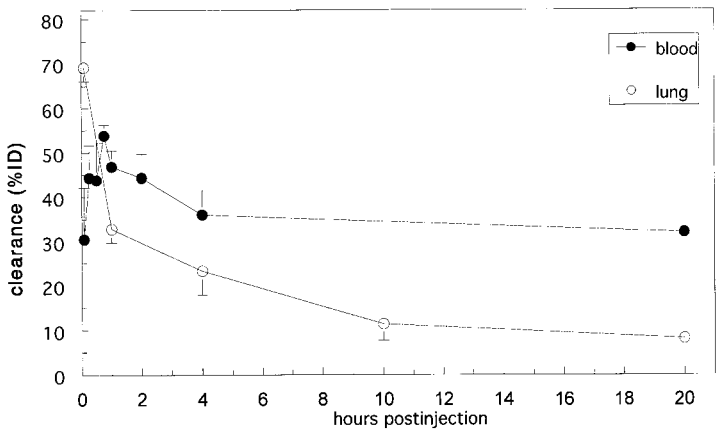


Figure 4. *In vivo* measurements of granulocyte function. Blood clearance of ^{111}In-leukocytes, calculated from serial blood samples (●). Lung clearance of ^{111}In-granulocytes, determined by quantitative analysis of scintigraphic images of rabbits injected with ^{111}In-granulocytes (◯). Error bars represent SEM.

44-h images as a function of time was statistically not significant (1.46 – 0.17 versus 1.50 – 0.30, respectively, and 1.53 – 0.14 versus 1.54 – 0.30, respectively; $p>0.05$). This correlated with the qualitative analysis of the scintigrams.
Quantitative analysis of the scintigrams of ^{111}In-granulocytes showed rapid initial lung transit indicating that the labeling procedure had not affected granulocyte function (Figure 4). In addition, granulocyte recovery at 45 minutes was >40% (Figure 4), confirming preserved granulocyte integrity [Peters, 1988].

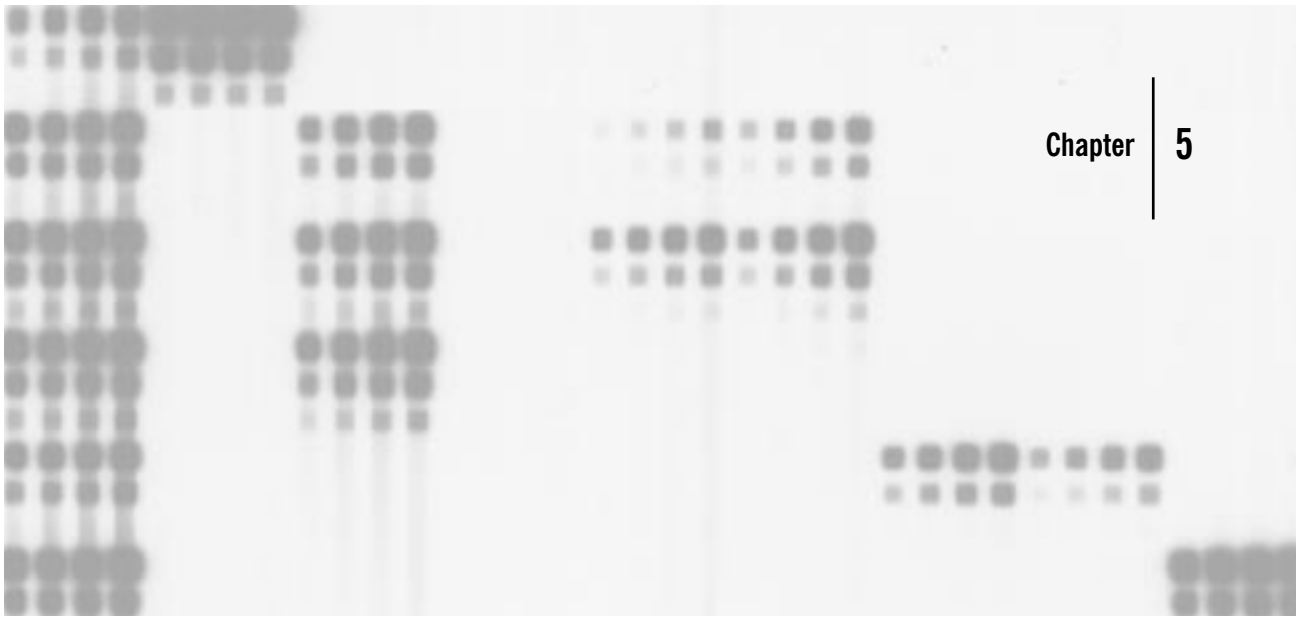
I Discussion

This study showed excellent performance by two new scintigraphic agents, ^{99m}Tc-IgG and ^{99m}Tc-PEG-liposomes, to visualize osteomyelitis. These new agents performed at least as well as ^{67}Ga-citrate and ^{111}In-granulocytes in the localization of chronic bone infection. Both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG have the major advantage of a ^{99m}Tc-label, providing a high photon flux at early time points after injection in combination with a low radiation dose, low costs, and continuous availability. In addition, both agents are easy to prepare and do not require handling of potentially contaminated blood. The lack of false-negative results for both agents refutes concern that a ^{99m}Tc-label would be less suited for the detection of low-grade osteomyelitis [Oyen, 1990]. Indeed, most infected rabbits were identified as early as 4 hours after injection. With ^{9m}Tc-PEG-liposomes as well as with ^{99m}Tc-HYNIC-IgG, all infectious sites were detected, whereas ^{67}Ga-citrate and ^{111}In-leukocytes each missed one case of osteomyelitis. Awashti *et al.* showed that radiolabeled liposomes were very effective in the evaluation of acute osteomyelitis in rabbits [Awasthi, 1998]. The target-to-nontarget ratios obtained with labeled liposomes in present study were slightly lower than those found in their study. This difference can be explained quite easily by the fact that in the latter study no sham procedure was performed. In addition, because focal accumulation of liposomes is thought to depend on increased vascular permeability, uptake of the agent is expected to be lower in chronic than in acute inflammation [Morrell, 1989]. The lower ratios apparently did not compromize accurate qualitative assessment, probably because the pattern of uptake of the radiolabel contributed to delineation of the infection.

^{111}In-labeled IgG has been shown to be very suitable for the evaluation of patients with musculoskeletal infections, including chronic osteomyelitis [Oyen, 1992d]. ^{99m}Tc-HYNIC-IgG has demonstrated equal efficacy to that of ^{111}In-IgG in this patient category [Dams, 1998a]. The results of our study confirm the ability of ^{99m}Tc-HYNIC-IgG to detect chronic, low-grade infection.
The high uptake of ^{99m}Tc-MDP at the osteomyelitis site did not result in improved infection-to-sham-surgery ratios compared to the other agents. In fact, the absolute uptake of the agent at the distal femur in the false-negative case was as high as 2 %ID/g. Therefore, despite high absolute uptake in the infected site, the ratio was only 1.07, illustrating the increased uptake of the agent at sites of bone repair for any cause. In contrast, for ^{67}Ga-citrate both absolute uptake and infection-to-sham ratio were similar to the values obtained with the other non-bone-seeking agents, indicating that accumulation of ^{67}Ga-citrate at the sham-surgery site was relatively low. Despite the similar uptake and ratios, ^{67}Ga-citrate and ^{111}In-leukocytes scintigraphy appeared to have lower sensitivity for the detection of infection than did ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG. This discrepancy probably resulted from the suboptimal imaging characteristics of both ^{67}Ga and ^{111}In.
The low yield of radiography in this study confirmed that this technique is unreliable in establishing infection in areas of violated bone [Elgazzar, 1995].
The use of labeled granulocytes in a rabbit model of chronic inflammation has its pitfalls. Because rabbit leukocytes are easily damaged by handling, the separation procedure could have negatively biased their performance [Lillevang, 1994]. In this study, we were able to establish preserved granulocyte function by means of *in vivo* tests, which are reliable markers of leukocyte damage [Peters, 1988]. The predominance of mononuclear cells such as lymphocytes and macrophages in areas of chronic infection raises the question whether the use of a granulocyte-enriched preparation in this model was justified. Indeed, some authors favoured the use of a mixed-cell preparation in chronic inflammation [Datz, 1994, Schauwecker, 1988]. More recently, however, it has been shown that ^{111}In-labeled lymphocytes actively eliminated the radiolabel, arguing against the concept that lymphocytes contribute to a positive image in chronic inflammation [Kuyama, 1997, Peters, 1996]. Using a granulocyte-enriched mixture, we maximized the activity made available to the infectious lesion, in accordance with the guidelines of Peters [Peters, 1996]. Still, the equivocal result in one infected rabbit was probably a result of the relatively mild infection in that animal.
In conclusion, in this rabbit model of chronic osteomyelitis, ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG performed at least as well as ^{111}In-granulocytes

and ^{67}Ga-citrate. Although the latter two agents each gave one equivocal result, labeled liposomes and labeled IgG correctly identified all infectious lesions. The absolute uptake and target-to-non-target ratios were very similar for the 4 agents. ^{99m}Tc-MDP gave one false-negative result because of high uptake at the sham-operated site, illustrating the nonspecific accumulation of this agent in areas of increased bone turnover. Because both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG display favorable dosimetric and physical characteristics and are easy to prepare, they can be valuable agents for the evaluation of chronic osteomyelitis.

Molecular diagnosis of musculoskeletal infection using PCR and reverse line blot



Chapter 5

I Introduction

Orthopaedic infections are often associated with the presence of biomaterials, like a joint prosthesis or osteosynthesis-hardware, since these increase the susceptibility to infection. In the presence of a foreign body, bacteria can alter their metabolism, which can result in a reduced growth rate. Some bacteria are also capable of producing a slime layer when adhered to the surface of a foreign body [Gristina, 1984, Gristina, 1983]. These changes in growth characteristics may not only hamper treatment, but can also complicate diagnosis of these infections using conventional (culture) techniques. Ultrasonication can disrupt adherent microbial biofilms and has been shown to increase detection of bacteria in prosthetic implant related infection [Gristina, 1985, Tunney, 1999, Tunney, 1998]. The long and tedious process of diagnosing mycobacterial infection is just another example that illustrates some of the limitations of conventional diagnostic modalities [Berk, 1996]. Osteoarticular tuberculosis frequently involves spinal infections and septic arthritis, but also tuberculous prosthetic joint infection has been reported [Berbari, 1998, Berk, 1996]. Depending on variables like the type of cultivation medium, the isolation of these bacteria from clinical samples takes 10 days to several weeks [Berk, 1996]. Contamination of cultures can occur when a long incubation period is required, like for *Propionibacterium acnes* [Calderone, 1996, Richards, 1995]. Fastidious or difficult to culture pathogens like *Bartonella henselae, Tropheryma whippelii, Mycoplasma pneumoniae, Ureaplasma urealyticum, Neisseria gonorrhoeae, Mycobacterium bovis and Mycobacterium ulcerans, Borrelia burgdorferi, Kingella kingae,* and *Chlamydia trachomatis* have been identified as etiological agents of osteomyelitis, and infectious or reactive arthritis [Altwegg, 1996, Hofer, 1993, Keret, 1998, La Scola, 1997, Lee, 1992, Liebling, 1994, Lin, 1999a, Oksi, 1994, Stahelin, 1998, Taylor-Robinson, 1992]. In addition, prolonged courses of antibiotic treatment can select for the small colony variant (SCV) type of growth of bacteria, with atypical clinical microbiologic characteristics that makes identification by culture difficult [Musher, 1977, Proctor, 1998, Proctor, 1995, Roggenkamp, 1998, von Eiff, 1997]. Species identification by phenotypic tests is also time-consuming and subjective [Fredricks, 1999]. Specific enrichments of the culture media or milieus, needed for the unknown pathogen to grow, are not known at forehand, and may not be applied routinely. Similarly, immunological detection of infection is only possible for the bacteria to which the immunolabels are directed [Tunney, 1999].

The idea that the accuracy of conventional microbiological modalities is not optimal for diagnosis of musculoskeletal infection, is supported by the fact that clinically evident infections sometimes remain culture-negative [Lyon, 1999]. Furthermore, empirically based treatment with broad-spectrum antimicrobials employed in culture-negative cases frequently resolves clinical signs of infection. Results from the Norwegian Hip Arthroplasty Registry showing that the rate of aseptic loosening of total hip prostheses decreased when antibiotic-containing bone cement was used for the primary implant, strongly support this approach [Espehaug, 1997, Havelin, 1995]. Thus, in some cases, the aseptic (based on negative culture outcomes) loosening of a prosthesis, might in fact have been caused by a low-grade infection or infection with fastidious bacteria [James, 1994]. An optimal diagnosis of the infection is essential, because reimplantation of a joint prosthesis in an infected implant bed is at great risk of failure due to recurrent infection. So, there is a need for detection techniques, which can bypass limitations of conventional microbiological modalities in diagnosing orthopaedic infections.

A promising alternative for conventional microbiological diagnosis methods is the use of molecular biological techniques. Deoxyribonucleic acid (DNA) was discovered already in 1869 by Friedrich Miescher, but it was the invention of the polymerase chain reaction (PCR) in 1983 by Kary Mullis that swung this field of research [Mirsky, 1968, Mullis, 1986, Mullis, 1990, Mullis, 1987, Saiki, 1985]. Genotypic identification of bacteria is possible by PCR, that rapidly amplifies specific regions of bacterial genomes, and subsequent molecular identification assays. The uniform requirement for PCR is only the presence of bacterial DNA, whereas cultivation conditions of diverse bacteria are variable. This allows for reduction in diagnostic delay, especially of slow growing or nonviable organisms, because the amplification of DNA does not depend on phenotypic growth characteristics. Thus, treatment strategies and antibiotic administration might be initiated earlier and more reliable aimed at specific pathogens, including uncultured and fastidious organisms.

In addition, PCR sensitivity has been reported to be extremely high, requiring only a small number of organisms (usually 10 bacteria or less). Due to the almost exponential multiplication of a single copy of template DNA, a PCR run for 30 to 40 cycles is capable of 10^6- to 10^7-fold amplification of one single copy. This feature might reduce the delay of antibiotic treatment and improve clinical outcome, when the treatment would otherwise have been postponed until intraoperative cultures are taken. Furthermore, an increase of diagnostic accuracy can facilitate diagnosis of clinical low-grade infection, like for example encountered in some patients with chronic osteomyelitis or

prosthesis infections. In this study, the reverse line blot hybridization (RLB) technique was explored as a means to identify the bacterial pathogens of musculoskeletal infection, based on the broad-range PCR amplification of the 16S ribosomal RNA (16S rRNA) gene sequence. This gene encodes the 16S (S stands for Svedberg unit, indicating sedimentation rate) subunit of rRNA that contains approximately 1545 nucleotides, and is commonly used as a target for broad-range PCR amplification of DNA products from multiple microorganisms [Conrads, 1997, Fredricks, 1996, Greisen, 1994, Jalava, 1996, Knox, 1998, La Scola, 1997, Mariani, 1995, Mariani, 1996, Relman, 1992, Sasaki, 1997, Wilson, 1990a]. The presence of such conserved sequences in the genomes of all known human pathogens in the eubacterial domain, but not in those of eukaryotic (including fungal and human) cells, allows for a differentiation between DNA of human tissue and that of bacteria [Lane, 1985, Relman, 1993]. Using RLB, it is not only possible to rapidly identify the amplicon of the highly conserved 16S rRNA gene, but also to discriminate between many eubacterial species, families, genera, and groups. This discrimination is based upon the variation of the 16S rRNA gene across the eubacterial domain that is present within the conserved sequences (Figure 1). RLB involves the use of complementary oligonucleotide probes that are tailored to target more or less variable regions of the 16S rRNA gene, to specify them for a species or a broader group of bacteria respectively. Previous PCR-based assays used a series of nylon membranes to which amplified DNA was attached that could be screened for hybridization with a labeled oligonucleotide probe [Saiki, 1989]. In a reverse manner, a set of

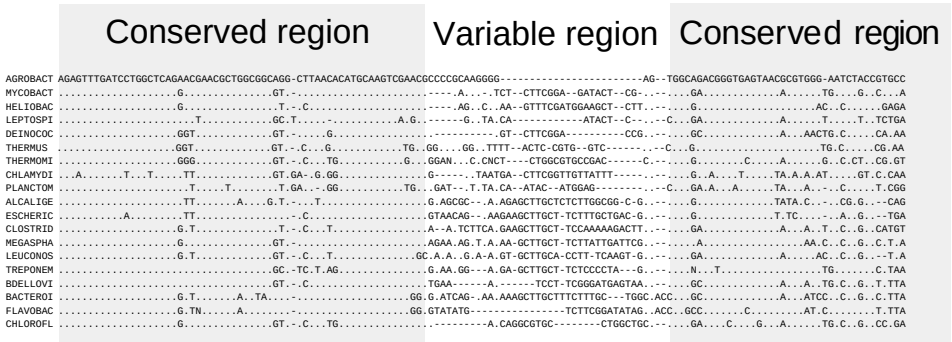


Figure 1. Alignment of different bacterial DNA sequences differentiates conserved regions containing identical nucleotides for all species and variable regions containing different nucleotides for each species.

oligonucleotides can be bound in parallel lines on a membrane, and screened for hybridization with labeled PCR products, hence the term reverse line blot [Kaufhold, 1994]. For the design of the primers and probes, information on 16S rRNA gene sequences can be obtained from public databases, available on the internet.

The aim of this study was to investigate the feasibility of the use of RLB as a diagnostic tool in orthopaedic infections. For this purpose, a set of oligonucleotide probes, based on bacteria typically encountered in orthopaedics, was designed, and tested for accuracy. Intraoperative tissue specimens of orthopaedic patients were used to evaluate diagnosis accuracy of RLB in comparison with subsequent culture results. Also, RLB was used complementary to standard cultures in the experimental studies described in chapters 10 and 11 on tobramycin-containing bone cement.

Materials and methods

Principle of reverse line blot

The principle of RLB is outlined in Figure 2. In one assay of RLB, up to 43 different oligonucleotide probes, attached in lines on a membrane, can be screened for reactivity on the same number of PCR amplification products. Broad-range 5 biotin-labeled 16S rRNA primers were used to set up the PCR. Species-, genus-, and group-specific oligonucleotide probes were designed to identify bacteria frequently seen in musculoskeletal infections. In addition, a generic eubacterial probe was used to screen for the presence of other bacterial species in the specimen.

Specimen collection

Accuracy and applicability of RLB was tested using bacterial lysates and surgical human tissue samples from orthopaedic patients. The latter were collected intraoperatively from patients undergoing orthopaedic surgery at the University Medical Center Utrecht, like primary total hip replacement, revision of infected or aseptically loosened prostheses, septic arthritis, and surgical treatment of pseudarthrosis. To reduce sample handling, tissue samples were immediately transferred in digestion buffer (500 mM Tris, pH 9, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml proteinase K) and stored at -70¡C until further processing. All molecular microbiological

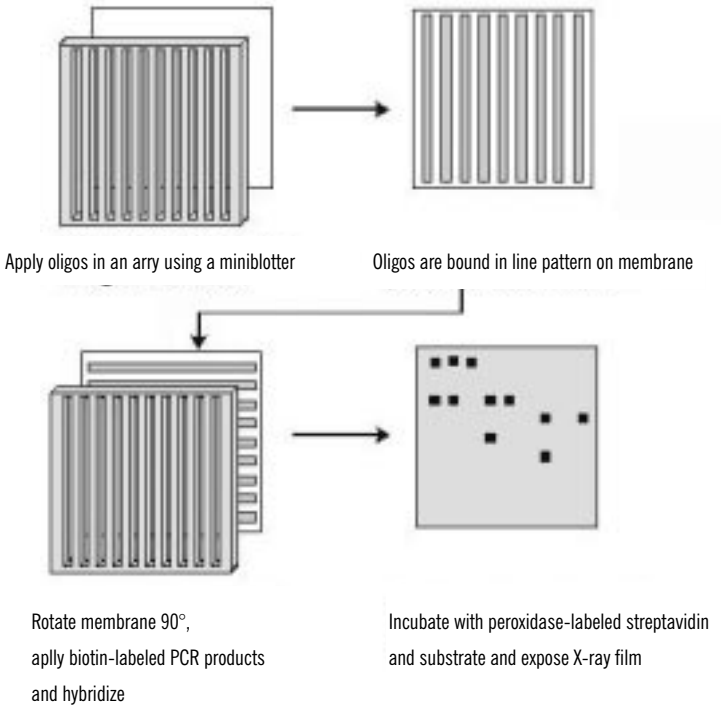


Figure 2. Principle of reverse line blot technique (Oligos=Oligonucleotide probes)

testing was performed at the Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. Specimens for culture were obtained and processed using standard protocols at the Eijkman-Winkler Institute for Clinical Microbiology, University Medical Center Utrecht, The Netherlands.

DNA extraction

The samples in digestion buffer were incubated for 18 hours at 60¡C to release total DNA. PCR fragments were purified using the QIAamp Tissue Kit (Qiagen, Hilden, Germany), according to the supplier s instruction.

PCR

For the 16S PCR, primers based on the highly conserved region of the eubacterial 16S rRNA gene were used (Table 1). DNA was amplified in 25-µl reaction volumes. Each reaction mixture contained 20 pmol of primer

Table 1. Set of oligonucleotide primers and probes used in PCR and RLB.

Oligonucleotide name	Sequence (5' -> 3')*	Target organism	Nucleotide position
Primers			
16S8FEvar1	CGGAATTCCAGAGTTTGATCMTGGCTCAG	Eubacteria	8-27
B-16S8RBvar2	5' biotin-CGGGATCCCGCTTTACGCCCARTNASTCCG	Eubacteria	556-575
Probes			
A-BORTMPA	5'-amino-TACAATCTAAGATCCAGACT	TmpA plasmid spike	
A-16SA339	5'-amino-TCCTACGGGAGGC(AT)GCA	Eubacteria	339-355
A-16SHIGHGC	5'-amino-GGTAGCCGGCCTGA	Gram+, High GC	247-260
A-16SLOWGC-2	5'-amino-GTGCCTAATACATGCAAG	Gram+, Low GC	45-62
A-16SPALPHA	5'-amino-GAGTGATGAAGGCCTTAG	Proteo group alpha	406-423
A-16SPGAMMA	5'-amino-AAGCCTGATGCAGCCAT	Proteo group gamma	383-397
A-16SSTAPH	5'-amino-AACCTACCTATAAGACTGG	Staphylococci	262-280
A-16SSAUREUS	5'-amino-TCAAAAGTGAAAGACGGTC	Staphylococcus aureus	334-352
A-16SCOR	5'-amino-CTTTAGTGTGTGTGGTGGA	Corynebacterium spp.	183-201
A-16SNEIS	5'-amino-AACATATCGGAACGTACC	Neisseriae	119-136
A-16SSALG	5'-amino-GCGGGGGATAACTATTG	Streptococci	142-158
A-16SSTREPD	5'-amino-CATGTTAGATGCTTGAAAGGAG	Streptococcus bovis group	197-218
A-16SSVIR	5'-amino-TGAGAGTGGAAAGTTCACAC	Streptococcus viridans and bovis	311-330
A-16SSPYO	5'-amino-TAACGCATGTTAGTAATTTAAAAG	Streptococcus pyogenes	144-167
A-16SPROT	5'-amino-GTTAATACCTTTATCAATTGACG	Proteus spp.	381-403
A-16SPRMIR	5'-amino-GGTTAATACCCTTATCAATTGA	Proteus mirabilis	380-401
A-16SECOLI	5'-amino-AAGGGAGTAAGTTAATACCT	Escherichia coli	451-471
A-16SMYC	5'-amino-GCYTGGGAAACTGGGT	Mycobacteria	146-161
A-16SMTUB	5'-amino-CGGGATGCATGTCTTGT	Mycobacterium tube rculosis	181-197
A-16SENTEROB	5'-amino-CTGATGGAGGGGGATAA	Enterobacteriaceae (partly**)	136-152
A-16SEAG	5'-amino-CCTGTCGATTGACGTTACC	Enterobacter/Pantoea agglomerans	101-119
A-16SPSA	5'-amino-TAATACCTTGCTGTTTGACG	Pseudomonas aeruginosa	462-481
A-16SPACNES	5'-amino-GAAAGGCCCTGCTTTTG	Propionibacterium acnes	41-5

*The underlined sequences represent the EcoRI and BamHI restriction sites.

**A-16SENTEROB targets various members of the Enterobacteriaceae, including the genera Escherichia, Enterobacter, Klebsiella, Serratia, Salmonella, and Yersinia.

16S8FEvar1, 160 pmol of primer 16S556RBvar2, 12.5 µl of HotStarTaq Master Kit (Qiagen, Hilden, Germany). A 25-µl overlay of paraffin oil added to the tubes. HotStarTaq Master Kit is a ready-to-use mixture of HotStarTaq DNA polymerase (0,1 U/µl), PCR buffer (containing 1.5 µM MgCl2), and nucleotides (200 µM of each dNTP), thereby reducing the risk of carryover (i.e. previous amplified products contaminating subsequent amplifications) during pipetting in the setup of the PCR. Nonspecific amplification during the setup of the PCR is prevented because the enzyme is inactive until incubation at 95 ¡C for 15 minutes. To further minimize contamination, PCR-mixtures were incubated for 1 hour at 37 ¡C with a combination of 3 restriction enzymes (CfoI, NciI, Sau3A1, Boehringer Mannheim GmbH, Mannheim, Germany 1.0 U/reaction) [Carroll, 1999, Conrads, 1997, Sharma, 1992]. The restriction enzymes were subsequently inactivated by incubation at 95¡C for 15 minutes, following which 5 µl of the purified DNA extract or template DNA was added and PCR amplification in an OmniGene Thermal Cycler (Hybaid, Teddington, Middlesex, UK) commenced. To minimize nonspecific amplification, a touchdown PCR program was used: two cycles of 20 s at 95¡C (denaturation), 1 minute at 66¡C (annealing), 1 minute at 72¡C (extension), and then two cycles with conditions identical to the previous cycles, but with an annealing temperature of 64¡C. During subsequent two-cycle sets, the annealing temperature was lowered by 2¡C until it reached 56¡C. Then, an additional 40 cycles, each consisting of 20 s at 94¡C, 1 minute at 56¡C, and 1 minute at 72¡C, following the touchdown program, was performed. The PCR was ended by an extra incubation for 7 minutes at 72¡C.

To monitor for the occurrence of false-positive PCR results, negative controls (digestion buffer without patient material) were included during extraction of the DNA samples. In addition, each time that the PCR was performed, negative control samples (reaction mixture without DNA template) were included. Furthermore, each PCR sample was spiked with a positive control (TmpA gene product cloned in E. coli plasmid, with built-in 16S rRNA gene primers) to detect inhibition of PCR (not shown in this chapter). In order to minimize contamination, the reagent setup, the extraction and sample addition, and the PCR and RLB were performed in three separate rooms, of which the first two rooms were kept at positive pressure and had airlocks.

Reverse line blot

The 5 terminal aminogroup of the oligonucleotide probes was used to covalently link them to an activated negatively charged Biodyne C membrane (Pall Europe Ltd., Portsmouth, United Kingdom). The membrane was activat-

ed by incubation for 10 minutes in 16% 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (Sigma), rinsed with water, and placed in a miniblotter (Immunetics, Cambridge, MA). Using the miniblotter, 25 to 3200 pmol of 5 amino-linked oligonucleotide probes dissolved in 500 mM NaHCO3 (pH 8.4) were coupled covalently to the activated membrane in a line pattern. After 1 minute of incubation, excess solution was aspirated, and the blot was inactivated by incubation with 100 mM NaOH for 10 minutes. Subsequently, the membrane was washed in 2x SSPE (360 mM NaCl, 20mM NaH2PO4, 2 mM EDTA) with 0.1% SDS at 60¡C for 5 minutes, and then stored at 4¡C or used immediately. The membrane was placed in the miniblotter with the oligonucleotide-containing lines perpendicular to the slots in the miniblotter. Ten microliter of the biotin-labeled PCR products were diluted in 150 µl of 2x SSPE-0.1% SDS, denatured for 10 minutes at 99¡C, and cooled rapidly on ice. After filling the slots of the miniblotter with the denatured PCR products, hybridization was performed for 1 hour at 42¡C. Next, following aspiration of the slots, the membrane was removed from the miniblotter and washed twice for 10 minutes each time in 2x SSPE-0.1% SDS at 55¡C. Subsequently, the membrane was incubated for 30 minutes at 42¡C with streptavidine-peroxidase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany) diluted 1:4,000 in 2x SSPE-0.5% SDS, and was washed twice for 10 minutes in 2x SSPE-0.5% SDS at 42¡C. Finally, the membrane was briefly rinsed twice with 2x SSPE. Hybridization was visualized by incubating the membrane with enhanced chemoluminescence detection liquid (Amersham International plc, Den Bosch, The Netherlands) and exposing the membrane to X-ray film (Hyperfilm; Amersham).

We designed 21 species-, genus- and group-specific oligonucleotide probes to identify bacteria frequently seen in orthopaedic infections (Table 1) [Bouza, 1999, Brause, 1998, Deacon, 1996, Vogely, 2000a]. In addition, a generic eubacterial probe was designed to detect bacterial species that were not identified by these specific probes. Sequences used to design these probes were derived from GenBank (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide) [Benson, 2000 #3109]. The Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov:80/BLAST/) was used to search for similarities between a query sequence and those contained in databases of the National Center of Biotechnology Information [Altschul, 1990]. Specificity control was also performed using the CHECK-PROBE function available with the Ribosomal Database Project facility (RDP, http://www.cme.msu.edu/RDP/) [Maidak, 2000]. The length of the oligonucleotides was adapted to ensure equal melting temperature (Tm) of the

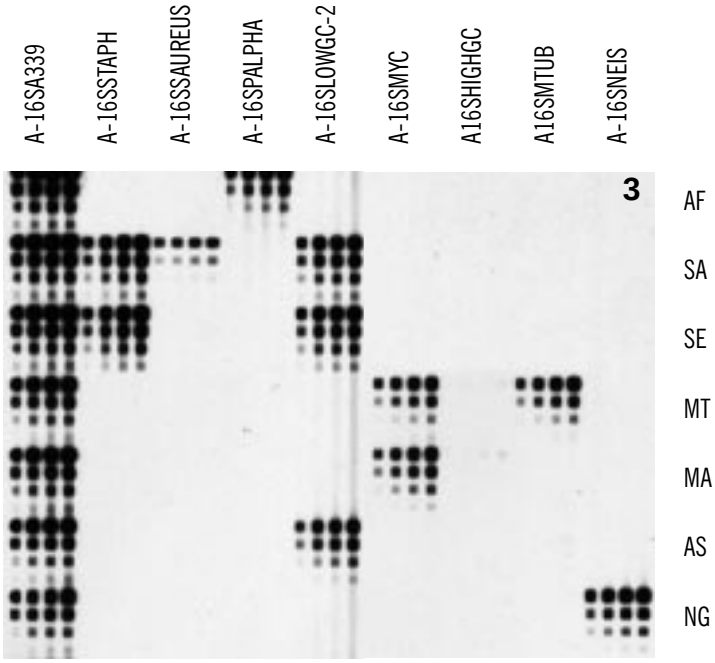
probes [Saiki, 1989]. In addition, care was taken to avoid internal loops that might influence hybridization efficacy. All probes were synthesized as 5 aminogroup bound to the oligonucleotide.

I Results

Figures 3-5 show some results of RLB in which lysates of several bacterial species, amplified with the broad-range 16S rRNA primers, were used to assess the accuracy of oligonucleotide probes. The generic eubacterial probe correctly detected all bacterial species tested. In addition, the identity of most species was correctly revealed by the group-, genus- or species-specific oligonucleotide probes. Probe A-16SMYC, named after the genus *Mycobacteria*, detected not only *M. tuberculosis* and *M. avium* (Figure 3), but also *Corynebacterium pseudotuberculosis* (not shown). The A-16SHIGHGC probe detected Gram-positives with a high %GC content, like mycobacteria, corynebacteria and *Propionibacterium acnes*, but hybridization signals were weak (Figure 3). Higher concentration of this probe resulted in false-positive outcomes (patient 8 in Figure 6, and patient 9). The A-16SSTREPD probe correctly detected streptococci, belonging to the bovis group, but also, incorrectly, *S. milleri* (Figure 5). However, the *S. milleri* group has been shown to be heterogenous, carrying members from several serogroups [Kilian, 1998]. Also in this figure, *Klebsiella pneumoniae* was misidentified as *Enterobacter agglomerans* by the A-16SEAG probe.

RLB confirmed the results of culture, being either negative or positive, in the majority of specimens from 21 patients undergoing orthopaedic(-related) surgery, presented in Table 2. In patient 16, two species were cultured from an infected total hip prosthesis (sporadic *Enterobacter cloacae* and sporadic *Enterobacter agglomerans*). Only the presence of the latter could be interpreted from the RLB outcome. The presence of both staphylococci and streptococci were correctly identified in specimens from an infected wrist (patient 21). A coagulase-negative staphylococcal strain was cultured in 1 of 3 specimens in patient 17, whereas RLB results were negative.

Specimens with extracted DNA of synovial fluid of 3 patients (18, 19, and 20) with septic arthritis, described in a previous study by Van der Heijden *et al*. (respectively patient 5, 1, and 3 in their study), were generously donated (B Wilbrink) and subjected to identification by RLB [van der Heijden, 1999b].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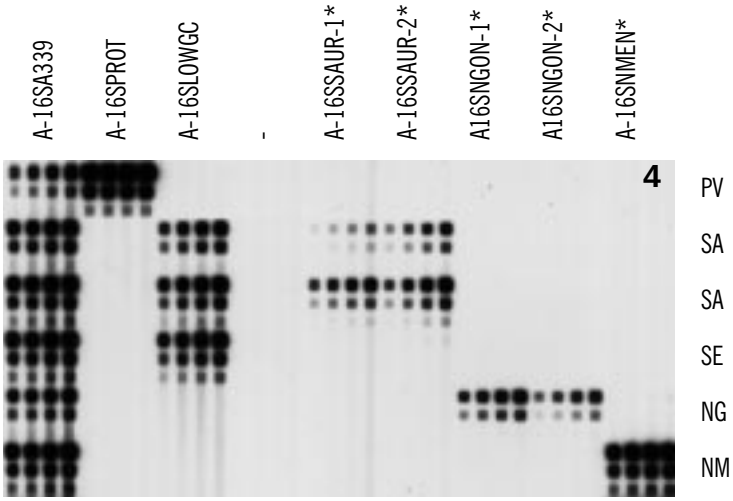
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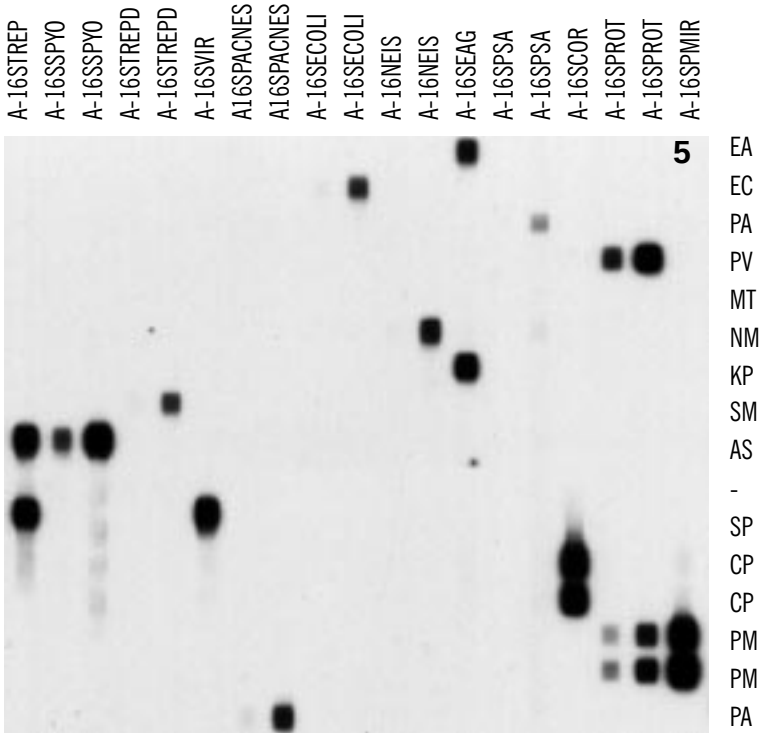
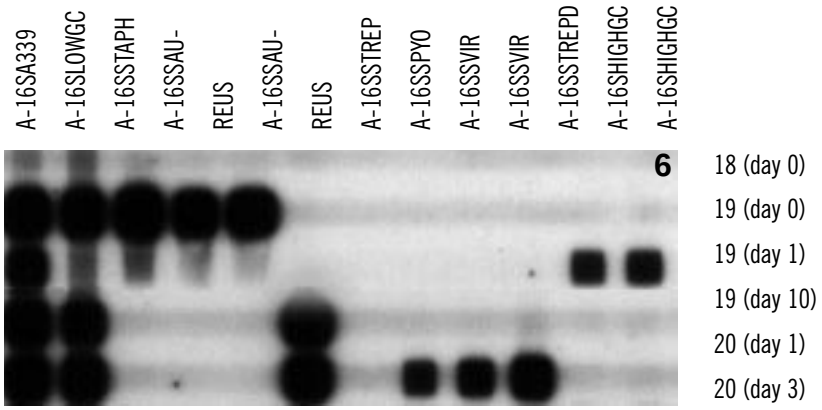


Figure 3-5. RLB outcomes using the following bacterial lysates (added horizontally): AF=A. felis, SA=S. aureus, SE=S. epidermidis, MT=M. tuberculosis, MA=M. avium, AS=group A streptococcus, NG=N. gonorrhoeae, PV=P. vulgaris, NM=N. meningitides, EA=E. agglomerans, EC=E. coli, PA=P. aeruginosa, KP= K. pneumoniae, SM=S. milleri, -=no lysate, SP=S pneumoniae, CP=C. pseudotuberculosis, PM=P. mirabilis, PC=P. acnes. Oligonucleotide probes were bound in vertical lines on the membrane (Some of these (*) were not used for clinical specimens, Figure 4) The amount of each probe per lane increased over 4 subsequent lanes from left to right, and the amount of each biotin-labeled bacterial PCR product decreased 10-fold per lane from top (25 ng = $3*10^9$ copies of PCR products) to bottom.(Figures 3 and 4). In Figure 5, some probes were added twofold, increasing in amount per lane from left to right.



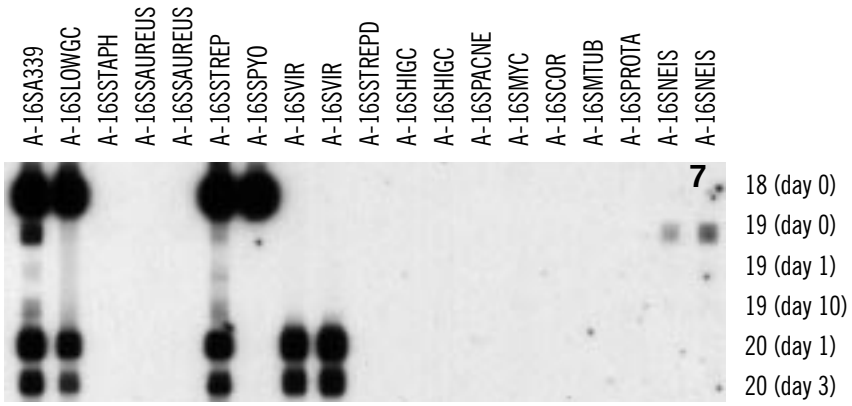


Figure 6-7. RLB outcomes using patient material (added horizontally). Patient numbers refer to those in Table 2. **Figure 6** includes samples taken from patient undergoing primary or revision total hip arthroplasty. **Figure 7** includes patient material obtained from intraarticular biopsies in septic joints before or after the start of antibiotic treatment.

Figure 7 shows that in the present study, RLB detected and identified the presence of bacterial DNA, confirming the results by the Van der Heijden *et al.*, obtained both by culture and by automated sequencing following universal 16S rRNA PCR of these specimens. In present study, hybridization of *N. gonorrhoeae* PCR products to the A-16SNEIS probe gave relatively weak signals on day 0, and negative results thereafter.

Serially diluted *S. epidermidis* spike DNA was used to test the sensitivity of RLB for the detection of PCR products. This experiment confirmed that the amplification of one copy of *S. epidermidis* chromosomal DNA resulted in a positive signal. The sensitivity was 100 to 1000 times higher as compared to ethidium bromide staining after gel electrophoresis (LM Schouls: personal communication).

Discussion

This study shows that it is possible to design oligonucleotide probes for RLB-based detection of bacteria encountered in musculoskeletal infection. Preclinical tests showed good accuracy in identification on group-, genus-, and species-level for most probes described in this study. Furthermore, the generic eubacterial probe could detect the presence of otherwise unidentified 16S rRNA gene amplification products. In addition, preliminary clinical testing showed that RLB could be used to detect pathogens in tissue specimens from patients.

Preclinical testing showed some hybridization errors of a few probes, The A-16SMYC probe was designed to detect *M. tuberculosis* and other mycobacterial species, and a separate study (LM Schouls, personal communication) to identify mycobacterial species showed that at least 25 different mycobacterial species could be detected using this probe. However, information derived from sequence databases also indicated matches with other closely related genera, like *Corynebacterium* spp., which was confirmed by this study. Based on homology searches, *S. milleri* and *K. pneumoniae* did not completely match the A-16SSTREPD and A-16SEAG probe sequences respectively, but cross-hybridization was detected in this study. *E. faecalis*, previously known as *Streptococcus faecalis*, was split from Streptococcus to form the genus Enterococcus, only in 1984 [Holmes, 1998]. Not surprisingly, this species hybridized to the A-16SSTREP probe (Table 2, patient 13).

Figure 6 shows an example of the detection of bacteria in clinical specimens using RLB. Both culture-positive and culture-negative results were confirmed by the hybridization assay. In addition, two RLB-positive results in culture-negative samples were noted. Firstly, hybridization of the sample from patient 9 with the A-16SHIGHGC probe suggests nonspecific reactivity, although it could not be excluded that Gram-positives in these clinical specimens were misidentified as staphylococci by cultivation. It proved difficult to optimize

Table 2. Results of culture and reverse line blot in patient material

Patient	Indication	Culture	RLB
1	Revision THP (aseptic)	-	-
2	Revision THP (aseptic)	-	-
3	Revision THP (aseptic)	-	-
4	Primary THP	-	-
5	Primary THP	-	-
6	Pseudarthrosis tibia	-	-
7	Revision THP (infected)	S. aureus	S. aureus
8	Revision THP (infected)	S. aureus	S. aureus
9	Revision THP (infected)	S. aureus	S. aureus and Gram+ High GC
10	Revision THP (infected)	S. aureus	S. aureus
11	Revision THP (infected)	S. aureus	S. aureus
12	Revision THP (infected)	Group C Streptococcus	Streptococci
13	Revision THP (infected)	E. faecalis	Streptococci
14	Revision THP (infected)	S. oralis	Streptococcus viridans (and bovis)
15	Revision THP (infected)	E. cloacae	Enterobacteriaceae
16	Revision THP (infected)	E. cloacae and E. agglomerans	E. agglomerans
17	Revision THP (infected)*	CNS	-
18	Septic arthritis knee	S. pyogenes	Day 0: S. pyogenes
19	Septic arthritis	Day -2: N. gonorrhoeae	Day 0: Neisseria
		Day -1: -	Day 1: Neisseria
		Day 0, 1, 10: Not done	Day 10: Neisseria
20	Septic arthritis	Day 1: S. pneumoniae	Day 1: S. pneumoniae
		Day 3: -	Day 3: S. pneumoniae
21	Septic arthritis wrist	S. aureus and Group G Streptococcus	S. aureus and streptococci

(* 3d exchange of antibiotic-containing bone cement beads)

hybridization conditions of this probe. Lower concentrations (<200 pmol/lane) resulted in insufficient signal after the standard 15-min exposure of the X-ray film; stronger signals were only obtained after overnight exposure. Higher concentrations increased the signal, but resulted also in nonspecific hybridization with other Gram-positives. Secondly, in patient 14, the A-16SSTREPD probe misidentified the *S. oralis* strain, cultivated from a tissue specimen obtained during revision hip arthroplasty. Unfortunately, the tube

containing the DNA extract may have become contaminated with *S. milleri* material. Indeed, positive hybridization signals like in this figure, but not of the before mentioned probe, correctly identified the *S. oralis* strain previously. The negative findings of culture in all but one specimen of patient 17 may explain the RLB-negative result in this case.

In present study, *S. pneumoniae* could be detected in a specimen obtained from a septic joint, three days after initiation of intravenous antibiotic therapy, with negative cultures at this time point (Figure 7, patient 20) [van der Heijden, 1999b]. Several authors recognized the fact that remaining nonviable bacteria or DNA remnants of bacteria in macrophages can create positive PCR results [Canvin, 1997, Koehler, 1998, van der Heijden, 1999b, Wilbrink, 1998, Wilkinson, 1999]. Otherwise, when antibiotic treatment may have decreased bacterial growth and limited detection by cultivation, positive PCR studies could still be obtained [Canvin, 1997, van der Heijden, 1999b, Wilbrink, 1998].

Currently, sequence databases like GenBank contain many 16S rRNA gene sequences, but the 16S rRNA gene sequence of many bacteria, particularly those from the environment, are still unknown. Thus, it can be expected that accuracy of RLB increases in the near future because design of oligonucleotide primers and probes can be more reliable evaluated for homology. However, it is impossible to design 16S rRNA probes specific to each bacterial species, because of close homology of some bacterial species and high variability between different subspecies. This feature underscores the importance of oligonucleotide probes that target phylogenetically more conserved regions of the eubacterial domain. These probes can confirm the presence or absence of DNA of otherwise unidentified bacterial species, by identifying their genus or group level. Negative hybridization results of other related species-specific probes, may suggest the identity the otherwise unidentified species even more. For instance, if the *S. aureus* species-specific probe does not detect a pathogen, identified as a member of the genus *Staphylococcus*, it is likely a coagulase-negative staphylococcal strain (Figure 3). To a certain extent, this information may indicate the virulence of the pathogen and allow for narrowing of the antibiotic treatment.

This study reflects the increasing interest in the possibilities of using techniques such as PCR for research on musculoskeletal disorders, as indicated by a number of introductory reviews on molecular based diagnosis that recently appeared in orthopaedic(-related) literature [Chu, 1997, Dietz, 1996, Hoeffel, 1999, Louie, 1998, Mariani, 1998, Rosier, 1999, Shore, 1994, Shore, 1995]. In orthopaedics, only a limited number of studies described the use of

DNA-based tools for diagnosis of infection. Initially, PCR-based studies were used primarily to detect specific bacterial pathogens in septic joints and to differentiate septic arthritis from non-infectious (rheumatoid) arthritis [Lee, 1992, Liebling, 1994, Taylor-Robinson, 1992]. Subsequently, genus-specific primers and broad-based 16S primers were used to screen for the presence of bacterial DNA of various origins in bone and joint infection [Girschick, 1999, Hoeffel, 1999, Levine, 1995, Mariani, 1995, Mariani, 1996, Rantakokko-Jalava, 2000, Tunney, 1999, van der Heijden, 1999b, Wilbrink, 1998, Wilkinson, 1999]. Similar formats have been used for the diagnosis of other bacterial disorders like keratitis, meningitis, and bacteremia [Carroll, 2000, Conrads, 1997, Dicuonzo, 1999, Goldenberger, 1997, Greisen, 1994, Jalava, 1996, Knox, 1998, Kotilainen, 1998, Kroes, 1999, Ley, 1998, Whelen, 1996].

The drawback of many current studies using PCR to detect infection is that they either are directed at a few specific organisms, or need time-consuming sequence procedures to reveal the identity of the bacteria. Species-specific PCR is less sensitive to carryover and reagent contamination than when broad-range primers are used to setup the PCR [Meier, 1993]. After separation by size and charge through agarose gel electrophoresis, the species-specific oligonucleotide bands can be readily visualized by either direct ethidium bromide staining or by the more sensitive method of Southern blotting, in which DNA probes can recognize transferred fractions on a membrane [Mariani, 1995]. However, other pathogens than the ones specifically primed and probed for cannot be detected. To amplify more bacterial sequences, a nested PCR using primer sets that simultaneously target multiple DNA sequences, has been used to detect putative pathogens in arthritis [Braun, 1997, Muralidhar, 1994]. For the screening of unknown pathogens in clinical samples, a species-specific PCR is less appropriate.

Another alternative molecular microbiological method for infection diagnosis is to sequence amplified DNA products [Rosenthal, 1995]. However, mixed infections cannot be readily detected without additional cloning of the microbial sequences before sequencing [Rantakokko-Jalava, 2000, Wilkinson, 1999]. Furthermore, some strategies to prevent carryover of 16S PCR products impair the sequence reactions. This has been described for the application of heat-labile uracil-DNA-glycosylase and the incorporation during amplification of dUTP instead of dTTP [Wilbrink, 1998].

Especially when a larger spectrum of bacterial pathogens can be encountered, like in orthopaedic infections, a broad-range PCR on the 16S ribosomal RNA gene is more suitable to detect DNA of any of those bacteria. Mariani *et al*. compared the results of culture with those of universal bacterial PCR in

patients with symptoms after total knee arthroplasty, indicative for biomechanical failure or infection of the implant [Mariani, 1996]. The presence of PCR products from bacterial origin was detected by Southern blot analysis in preoperative aspirates from 32 patients. Of these, preoperative culture results were positive in only 6 samples and intraoperative culture results in another 9 patients. In contrast, all patients with a negative preoperative PCR result also had negative intraoperative cultures. Infections caused by different bacterial pathogens were detected using a eubacterial probe, but these authors did not identify the bacterial species from which the amplified DNA originated. The use of species-specific primer sets for this purpose was reported in a subsequent study by these authors [Mariani, 1998]. Species-specific oligonucleotide probes can also be used to analyze broad-range amplification products in Southern blot or a dot blot (in which multiple DNA samples are blotted as dots on a nylon membrane) hybridization formats [Dore, 1998, Krimmer, 1999]. This method has been used in infection diagnosis of patients with meningitis [Dicuonzo, 1999, Greisen, 1994]. However, such formats become laborious with an increasing number of clinical samples, or when many oligonucleotide probes are required for hybridization [Dicuonzo, 1999].

Detection formats in which a panel of oligonucleotide probes is immobilized on a solid support, like in RLB, offers the possibility to screen for hybridization of a clinical sample to multiple probes in a single run. Initially, this technique was employed as a reverse dot blot assay to detect allelic variants of human genes [Saiki, 1989]. The principal of reverse hybridization has also been applied for the detection and identification (genotyping) of viral, fungal, and bacterial species in so-called line probe assays (LiPA) [Kleter, 1999, Martin, 2000, Miller, 2000, Stuyver, 1996, van Doorn, 1999]. In LiPA, a nitrocellulose membrane strip, onto which oligonucleotide probes are immobilized as parallel lines, is used to detect hybridization of amplification products. Unlike RLB, the above-mentioned hybridization formats cannot be used to hybridize multiple test samples simultaneously. In contrast, RLB can simultaneously detect and identify multiple bacterial species in different samples [Bunschoten, 2000, Gubbels, 1999, Jacobs, 2000, Kaufhold, 1994, Rijpkema, 1995, Schouls, 1999, Sparagano, 1999, van der Heijden, 1999a]. In addition, unlike RLB membranes, LiPA strips are not reusable because hybridization on the latter is visualized by a color reaction. RLB has been helpful in large-scale epidemiological studies on mycobacteria, using probes for spacer regions in the mycobacterial genome (spoligotyping) [Aranaz, 1996, Kamerbeek, 1997, van Embden, 2000]. An upcoming technique that resembles RLB is the use of

DNA oligonucleotide arrays on silicon microchips. These so-called DNA chips can be used to screen for hybridization of DNA samples to thousands of oligonucleotide probes attached on a glass slide [Fredricks, 1999, Lipshutz, 1999]. In contrast to RLB, dedicated equipment is needed to make and read these microarrays [Cheung, 1999].

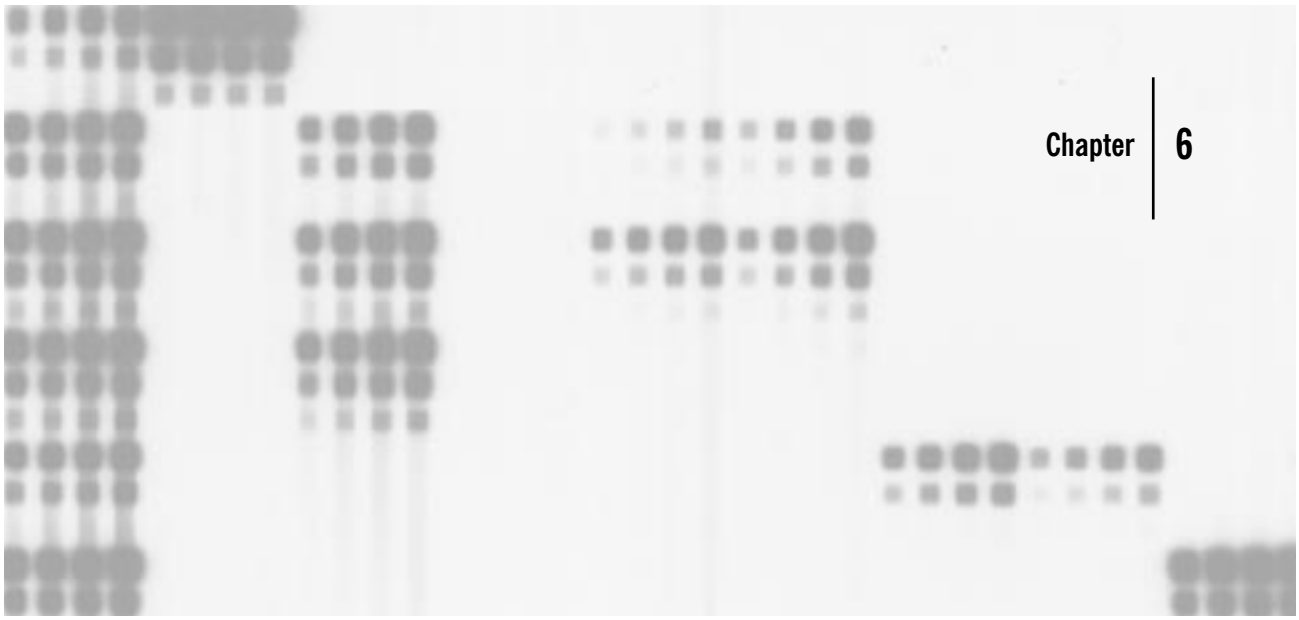
In the present study, broad range oligonucleotide primers specific for the conserved regions of eubacterial 16S rRNA gene were used to setup the PCR. One of the major problems of using this gene as an amplification target is contamination of reagents with bacterial DNA, since these reagents may be derived from bacterial sources [Corless, 2000]. In addition, contamination of exogenous DNA is possible during the setup of the PCR. Several methodologies have been described to overcome this problem, which is exacerbated by the highly sensitive nature of PCR [Kwok, 1989]. Many of these, such as the use of dedicated equipment (pipettes with disposable filter tips, disposable gloves and laboratory coats, nonreusable waste container bags) in dedicated rooms (with biosafety hoods equipped with ultraviolet germicidal lamps) for the extraction of DNA and the setup of the PCR, were used in the present study. Hoeffel *et al*. developed genus-focused primers for PCR-based detection of periprosthetic infection, that would amplify DNA of some Gram-positive cocci, but not of Escherichia coli [Hoeffel, 1999]. These primers were chosen because Taq polymerase enzyme may contain remnants of the latter bacterial species, in which it is recombinantly produced. Some treatments, like for instance ultraviolet (UV) irradiation, are highly effective in eliminating contaminating DNA, but result in an unacceptable (up to a 4- to 7-log) reduction in PCR sensitivity, due to inactivation of reagents. Thus, to improve accuracy of molecular diagnostic modalities, much research is focused on the development of PCR techniques that could decrease bacterial contamination, while maintaining high sensitivity for gene amplification.

In the near future, PCR-based diagnosis of infection will certainly complement conventional diagnostic modalities. Because RLB offers a simple and quick strategy to identify multiple bacterial PCR products after a broad-range PCR on the 16S rRNA gene, it may become an important tool for infection diagnosis. This approach allows for the detection of many different bacterial pathogens encountered in musculoskeletal infection, both in unibacterial and mixed infection, independent of growth characteristics. RLB, using the oligonucleotide probes described in this study, may also be applicable in diagnosis of other types of infections, caused by similar pathogens.

Our preliminary study indicates the feasibility of the RLB technique for use as a diagnostic tool for (biomaterial-associated) orthopaedic infections. At this

moment, testing is in progress to further optimize the reverse line blot technique for application in the diagnosis of orthopaedic infections. Testing a greater number of bacterial lysates may reveal flaws in the design of some oligonucleotide probes, or may confirm the accuracy of these in different strains belonging to the same species, genus or taxonomic group of bacteria. Fine-tuning of the dose of some oligonucleotide probes and identifying optimal stringency conditions are the next steps in upgrading this technique.

Introduction to experimental studies on antibiotic-containing bone cement



Chapter 6

Antibiotic-containing bone cement

In **Part II** of this thesis, pre-mixed tobramycin-containing bone cement was evaluated in experimental studies. In numerous hospitals, especially in the United States, antibiotic-containing bone cement is prepared by adding tobramycin powder intraoperatively to bone cement and subsequent hand-mixing [Heck, 1995) Pre-mixed antibiotic-containing bone cements are marketed in Europe, but regulations of the Federal Drug Agency (FDA) do not allow the use of these cements in the United States (U.S.). Although registration regulation for new bone cements have become less strict recently, both in Europe and in the U.S., strict regulations for manufacturing of bone cements should guarantee constantly reproducible manufacturing conditions and the safe use of these products clinically [K hn, 2000) Therefore, it is somewhat inconsistent that U.S. regulations allow less constant reproducible manufacturing conditions, i.e. the hand mixing of antibiotics and bone cement in the operating room by the surgical team. This hand mixing of antibiotics with bone cement does not allow for a consistent and controllable quality of the cement. A standardized preparation of antibiotic-containing bone cement benefits not only future infection-based efficacy studies, but also the evaluation of its biomechanical properties. Although adding low quantities (approximately one gram per unit) of tobramycin has minimal effects on the fatigue life of cement, amounts of more than two gram of antibiotics per unit of cement have shown to decrease the strength of cement [Davies, 1991, Lautenschlager, 1976].

Tobramycin

Since 1969, various combinations of antibiotics and bone cement have been used in the prevention and treatment of arthroplasty infections [Buchholz, 1970, Elson, 1977a, Murray, 1984]. Tobramycin, developed in 1968, is one of the aminoglycoside antimicrobial agents. Other members of this group are gentamicin, streptomycin, kanamycin, amikacin and netilmicin. Like other aminoglycosides, tobramycin is heat-stable, which makes it suitable for incorporation in polymethylmethacrylate (PMMA). Aminoglycosides are effective

primarily against most staphylococci, certain mycobacteria and aerobic Gram-negative bacilli such as enterobacteriaceae or *Pseudomonas aeruginosa* [Edson, 1999, Wingard, 1991]. The antimicrobial spectrum of tobramycin includes pathogens like staphylococci and aerobic Gram-negative bacilli, which are most frequently cultured from orthopaedic implant infections [Scott, 1999]. Aminoglycosides are bactericidal by binding to bacterial ribosomes and interfering with protein synthesis, and by disrupting the cell membrane. Differences in structure of the compounds determine the spectrum of activity against bacteria, the risk of toxicity in patients and occurrence of resistance. Aminoglycosides enter the inner cell membrane of the bacteria through an energy-dependent, aerobic process. Binding to the 30S-ribosomal subunit inhibits protein synthesis and induces misreading of the mRNA. These effects, together with cell membrane-related effects cause bacterial cell death [Edson, 1999, Greenwood, 1998, Lorian, 1996]. Antibacterial activity of aminoglycosides is concentration dependent, meaning that an increased drug concentration will enhance the bactericidal activity [Lode, 1998]. The respiratory processes needed for aminoglycoside uptake are absent in anaerobes, streptococci and enterococci, resulting in relative resistance [Greenwood, 1998]. Due to the high polarity of aminoglycosides, these compounds do not enter phagocytic cells and thus they are not active against intracellular bacteria [Greenwood, 1998, Wingard, 1991]. Resistance mechanisms involve most commonly aminoglycoside-modifying enzymes. These enzymes are plasmid-mediated, which means that this genetically information can spread easily to different bacterial species [Wingard, 1991]. The structure of a particular aminoglycoside determines its inactivation by the enzymes. Aminoglycosides can share some structural groupings vulnerable to modifications, which explains the emergence of cross-resistance between gentamicin and tobramycin. In a recent study however, more than half of gentamicin-resistant isolates of Enterobacteriaceae showed susceptibility for tobramycin [Adwan, 1998]. Susceptibility studies have shown that tobramycin is generally more active *in vitro* against most strains of *P. aeruginosa*, and gentamicin is more active against *Serratia* species [Edson, 1999]. Resistance rates of *P. aeruginosa* strains have been reported to be 7-14% for gentamicin *versus* 4% for tobramycin [Edson, 1999, Lorian, 1996]. but other authors found no difference between the two (18%) [Schmitz, 1999b]. However, data reported in literature on aminoglycoside susceptibility are of limited value for the surgeon with respect to the choice of a specific type of antibiotic-containing bone cement in the individual patient. Resistance to aminoglycosides, just as to other antibiotics, varies by location and local

usage patterns that can change over time [Schmitz, 1999a]. This is illustrated by the fact that - despite close association of aminoglycoside resistance with methicillin resistance [Schmitz, 1999b] - restricted use of gentamicin has been shown to induce reemergence of gentamicin-susceptible methicillin-resistant *S. aureus* [Aubry-Damon, 1997]. Therefore, is more prudent to rely on data, if available, specific to the hospital where antibiotic-containing bone cement is to be used for prevention of prosthesis infection. When an infected arthroplasty is being revised and culture results are known, the selection of the antibiotic-containing bone cement should be tailored to the causative pathogens.

Carriers of antimicrobials for local drug delivery

In **Chapter 7**, antimicrobial-loaded biomaterials are discussed that can be implanted locally for the prophylaxis or treatment of musculoskeletal infection. Each of these carriers, of which tobramycin-containing bone cement is just one example, has its specific indications for use in the management of these infections, because properties of these materials, such as form and biodegradability, vary. The multitude of antibiotics and other antimicrobial substances that are incorporated in these carriers, together with the material properties, determine the drug-release profiles that can be found either *in vitro* or *in vivo*.

Release of tobramycin from tobramycin-containing bone cement

The efficacy of antibiotic-containing bone cement in preventing or treating arthroplasty infection depends on the antibiotic release characteristics. In **Chapter 8**, the release of tobramycin from pre-mixed tobramycin-containing bone cement in the rabbit s femur was investigated. In order to be effective in preventing or treating arthroplasty infection, antibiotic-containing bone cement should release the antibiotic in concentrations above the susceptibili-

ty level of the microorganism involved. Secondly, this concentration should be reached at the site of the implant, especially at the bone-cement interface and in the surrounding bone. In addition, the antibiotic concentration in serum should not exceed toxic levels, to limit risk of side effects. Therefore, both serum and bone concentrations of tobramycin were investigated in the rabbit model described in this chapter.

I Susceptibility of bacteria to tobramycin

Still, it is difficult to translate results from susceptibility tests and release studies to clinical efficacy of antibiotic-containing bone cement. Several factors may influence the effect of antibiotics released from bone cement on the bacteria. This complicates the prediction whether the use of local antibiotic-containing bone cement will prevent or inhibit bacterial growth in the setting of implant infection. Firstly, concentrations of antibiotics that are higher than the minimum inhibitory concentration (MIC), will theoretically eliminate bacteria, defined as susceptible to these antibiotics [Johansson, 1991]. However, when initial release of antibiotic is high, even bacteria that are defined as resistant, based on their MIC, may be killed [Scott, 1999]. Secondly, in device-related infection, standard susceptibility tests do not correlate with treatment success [Blaser, 1995]. The microenvironment in arthroplasty infection might favor bacteria, as it includes the presence of foreign bodies and eventually devitalized bony due to previous surgical procedures or to the infectious process itself. In such a setting, bacteria can alter their phenotype, rendering them less susceptible to local defense mechanisms and immune responses of the body and to antibiotics. This phenomenon has been described for different biomaterials especially in relation with the capability of some bacteria to produce a polysaccharide biofilm on the surface of the implant. These phenotypic alterations *in vivo* may not be detected by standard susceptibility tests, since these tests measure *in vitro* phenotypic susceptibility of bacterial isolates [Johansson, 1991]. Gristina showed that despite normal MIC levels of tobramycin in broth suspension, exposure to higher levels of tobramycin of *Staphylococcus epidermidis*, enclosed in a biofilm on a stainless steel implant, did not result in a total reduction of viable bacteria [Gristina, 1987]. Similar results were obtained by Nickel *et al*. after exposure of *P. aeruginosa*, growing as a biofilm on urinary catheters, to

tobramycin [Nickel, 1985]. Other *in vitro* studies showed that gentamicin or tobramycin, impregnated in PMMA bone cement, could not completely prevent biofilm formation after incubation with *S. epidermidis* [Chang, 1991, Chang, 1992, Oga, 1992]. However, the addition of aminoglycosides to the PMMA reduced adherence and viability of these microorganisms [Chang, 1991, Chang, 1992, Oga, 1992]. In fact, in contrast to plain bone cement, the addition of gentamicin prevented infection after implantation of PMMA, preincubated with *S. epidermidis* [Chang, 1994, von Eiff, 1998]. Darouiche *et al*. showed that *S. epidermidis*, grown on stainless steel nuts in presence of vancomycin, could not be eradicated completely, even though high levels of vancomycin were reached in the biofilm [Darouiche, 1994]. Binding of antibiotics to glycocalyx material and consequent inhibition of diffusion is not a major mechanism of antibiotic resistance in biofilms, as has been shown for tobramycin and vancomycin [Darouiche, 1994, Nichols, 1988]. More likely, an altered physiology and growth rate of the bacteria diminish the antimicrobial effect in the biofilm environment [Darouiche, 1994, Nichols, 1988]. Another aspect of the problem of extrapolating laboratory results to the clinical situation, which has been described as the minefield of difficulties , is related to aminoglycoside-containing polymers: [Greenwood, 1981]. Von Eiff has shown that in the presence of gentamicin-containing beads, *S. aureus* can transform into so-called small colony variants (SCVs). These SCVs show a decreased growth rate, are therefore more resistant to antibiotics, especially to aminoglycosides, and may be easily missed in the clinical laboratory [Proctor, 1998, Proctor, 1995, von Eiff, 1997, von Eiff, 1998]. Subsequently, testing of susceptibility of only the parent strain may give misleading results [Proctor, 1998, von Eiff, 1997].

I Tobramycin-containing bone cement and prophylaxis of infection

Thus, the efficacy of antibiotic-containing bone cement to prevent or treat arthroplasty infection cannot be extrapolated just from susceptibility studies. Therefore, we performed various animal studies to evaluate the efficacy of the new tobramycin-containing bone cement. Firstly, in **Chapter 9**, the efficacy of this bone cement was evaluated for the prevention of local *S. aureus* and *S. epidermidis* infection. These bacterial species were used because they

are most frequently isolated in arthroplasty infection. Incidence rates after total joint arthroplasty of *S. epidermidis* (26-38% of infections) are somewhat higher than those for *S. aureus* (16-24% of infections) and have increased over the last decade [Fitzgerald, 1994, Garvin, 1993, Ostendorf, 2001, Sanzen, 1988, Tsukayama, 1996].

In literature, the optimal mode of administration of antibiotics - prior to or during surgery - is still subject of discussion. Dutch guidelines state that there is no indication for the use of antibiotic-containing bone cement in primary arthroplasty, if operated under prophylaxis of systemic antibiotics and an ultra-clean air system [Dutch Institute for healthcare improvement, 1994]. Data from the Swedish hip arthroplasty registry show however an increased use of antibiotic-containing bone cement in primary hip arthroplasty from approximately 10% of all primary hip arthroplasties performed in 1978 to 80% in 1996 [Swedish-National-Hip-Arthroplasty-Registry, 1998]. The different modes of administration of antibiotics have been compared for efficacy by only a few experimental and clinical studies. Petty *et al*. showed in a study in dogs that systemic antibiotic treatment as well as local treatment with antibiotic-containing bone cement reduced infections of the implant bed, but only the latter was found to be significantly different from controls [Petty, 1988]. Josefsson *et al*. compared prophylaxis with systemic antibiotics versus gentamicin bone cement in total hip arthroplasty in a prospective randomized clinical trial [Josefsson, 1990, Josefsson, 1993]. At 5 years follow-up, significantly more infections occurred in the group receiving systemic antibiotics. However, at 10-years follow-up of 1688 hips, infection rates in the systemic antibiotics group and in the antibiotic-containing bone cement group were no longer significantly different. In a similar study, McQueen *et al*. found no difference between these two modes of infection prophylaxis in 401 patients, at two years follow-up [McQueen, 1990].

It is obvious from the few available studies that there is still a lack of scientific proof regarding the efficacy of systemic (intravenous) and local (bone cement) administration of antibiotics to prevent implant bed infection. In **Chapter 10**, we evaluated the efficacy of either intravenous cefazolin or tobramycin-containing bone cement in preventing experimental implant infection. Cefazolin was used for systemic administration, since it is used widely by orthopaedic surgeons for treatment and prevention of staphylococcal infections.

I Tobramycin-containing bone cement and treatment of infection

In revision surgery, antibiotic-containing bone cement is often used, since revised implants become more frequently infected than primaries [Fitzgerald, 1977, Nasser, 1992, Wilson, 1990b]. The higher infection rate is not only the result of another operation in compromized tissue, but the previously mentioned difficulties in diagnosing implant infection may play their role as well. When an implant is inserted in an implant bed that has not been totally cleared from bacteria, the second implant is likely to become infected too. In case a prosthesis is infected, rigorous treatment modalities are necessary to eliminate the infection, but the best choice for treatment of an infected total joint prosthesis still remains to be clarified. Consensus exists among most orthopaedic surgeons to remove the infected prosthesis if possible, because the infection is difficult to treat in presence of foreign material covered with bacteria [Brandt, 1997]. Such a revision operation can be performed either as a one-stage procedure or as a two-stage procedure. In the one-stage revision, the infected implant is removed and, after debridement and lavage of the implant bed, replaced by a new prosthesis during the same session. In a two-stage revision, the insertion of the new implant is postponed until several weeks after removal of the infected implant. During this period, the infection is treated with systemic antibiotics and/ or local antibiotic-containing beads. The new prosthesis is inserted not until the infection parameters have regained normal levels. The use of antibiotic-containing bone cement for fixation of the revision prosthesis is preferred, given the higher incidence of infection after revision in comparison with primary joint prostheses. Where most surgeons choose the two-stage procedure for exchange of an infected prosthesis, also large series have been reported using the one-stage procedure, especially in Europe [Buchholz, 1981, Raut, 1995]. So far, reviews of literature on arthroplasty infection reported success rates of 82-83% for one-stage revisions, and 91-93% for two-stage revisions [Garvin, 1995, Pagnano, 1997]. Clinically, direct comparison between a one-stage or two-stage revision of a total joint prosthesis is difficult because of the lack of prospective, randomized studies evaluating the timing of this procedure [Garvin, 1995]. Clearly, the initial costs of a one-stage procedure as opposed to the two-stage procedure are less. Fewer operations means also less inconvenience for the patient. These advantages must be weighed against the risk of a re-infection of the prosthesis since frequently a second operation is needed anyhow. Together

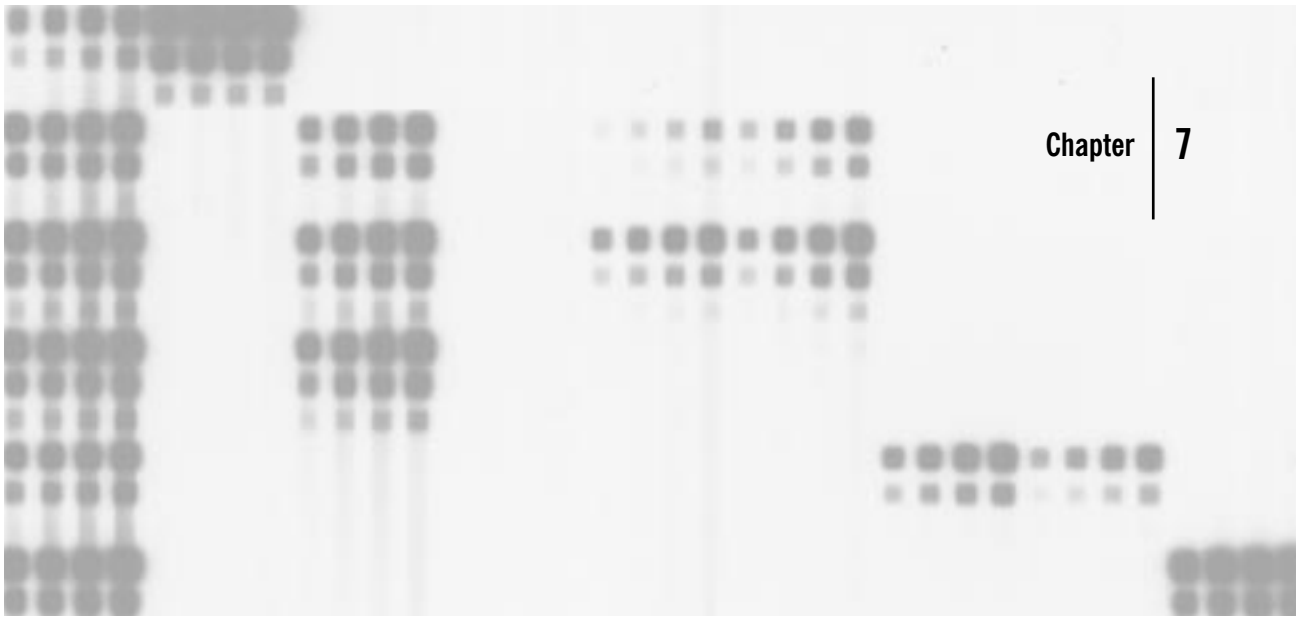
with the increase of the number of revision procedures a patient will undergo, there is an increased risk of complications due to bone loss and of potentially fatal outcome. The risk of a re-infection after the first revision procedure, rather than the costs associated with the initial procedure itself, is probably the major factor in comparing the cost-effectiveness between the one- or two-stage procedure. Comparative trials between both options have been advocated for to clarify this matter [Gillespie, 1997]. The need for long-term follow-up in the evaluation of infection rates has also been emphasized [Raut, 1995]. Some consensus exists on the indications for a one-stage revision. Infections caused by less virulent bacteria, fully susceptible to the antibiotics used for treatment, in otherwise healthy patients who lack additional risk factors for infection (rheumatoid arthritis, diabetes, decubitus, need for bone grafting) can probably be treated safely and effectively by a one-stage procedure. Using these criteria, two small series (respectively, 15 patients with a mean follow-up of 53 months and 20 patients with a mean follow-up of 10 years) reported no recurrence of infections [Mulcahy, 1996, Ure, 1998]. Altered circumstances (e.g. the emergence of resistant bacterial strains as the causative organisms in infected arthroplasties) can pose the surgeon to choose for a two-stage procedure, even when he favors the one-stage revision [Elson, 1994]. Still, the criteria for one- or two-stage revisions are subject to debate. For instance, Raut argued that a discharging sinus is not always a contraindication for a one-stage revision of an infected hip prosthesis [Raut, 1994]. The outcome of one-stage revisions of infected total knee prostheses has been reported to be inferior to those of hip prostheses, probably due to lower vascularity and minimal soft-tissue coverage of the knee [von Foerster, 1991].

To prevent a recurrent infection after implant revision, antibiotic-containing bone cement should be able to treat an already established osteomyelitis. This can be investigated in a one-stage revision model, since it lacks the temporarily treatment with antibiotic-containing beads or a spacer that could reduce the infection. Therefore, in **Chapter 11**, a one-stage revision model was used to test the efficacy in treating an infection with either tobramycin-containing bone cement or systemic cefazolin.

Aims of Part II of this thesis:

- To review the literature on antimicrobial-loaded carriers that are clinically available or are being developed for use in the management of musculoskeletal infection (**Chapter 7**)
- To investigate the *in vivo* release of tobramycin in blood and bone as a function of time, after insertion of premixed tobramycin-containing cement. (**Chapter 8**)
- To investigate the efficacy of premixed tobramycin-containing bone cement in the prevention of *S. aureus* and *S. epidermidis* infections. (**Chapter 9**)
- To investigate the efficacy of both premixed tobramycin-containing bone cement and systemic cefazolin in the prevention of *S. aureus* infection. (**Chapter 10**)
- To investigate the efficacy of tobramycin-containing bone cement and systemic cefazolin for treatment of *S. aureus* infection in a one-stage revision model. (**Chapter 11**)

Review on antimicrobial-loaded carriers in musculoskeletal infection



Chapter 7

I Introduction to antimicrobial-loaded carriers

Part of this thesis describes experimental studies that were performed using a new premixed tobramycin-containing polymethylmethacrylate (PMMA) bone cement. This antibiotic-containing bone cement is just one example of the variety of carriers with different kinds of antimicrobials that has been studied *in vitro* or *in vivo* for prophylactic and therapeutic purposes in musculoskeletal infection, as illustrated in Table 1. Although some carriers can be used for different types of osteomyelitis, their indication for use depends primarily on the form and characteristics of the material. A major indication for the use of antibiotic-containing bone cement in its injectable form lies in the management of arthroplasty infection. However, also the use of antibiotic-containing PMMA beads are widely used to treat osteomyelitis locally. To put the use of antibiotic-containing bone cement in a broader perspective, an overview of the available literature on antimicrobial-carriers used in orthopaedics is discussed below. Many experimental and a smaller, but increasing, number of clinical studies on these materials have been published. Some of those antibiotic-carriers are or will become available for clinical use by the orthopaedic surgeon, and it will be interesting to see whether this will influence the current role of antibiotic-containing PMMA bone cement in the management of orthopaedic infections.

I Bone graft

One of the first carriers to which antibiotics were added was bone graft. In 1946, Prigge reported some success after the use of autologous cancellous bone grafts to chronic osteomyelitic defects after local instillation with penicillin [Prigge, 1946]. In 1947, De Grood mixed these bone chips with penicillin before filling osteomyelitic defects [de Grood, 1947]. In the following decades, only a few studies on antibiotic-loaded bone graft, either allogeneic or autologous, have been reported [Durig, 1986, Lindsey, 1993, McLaren, 1988, McLaren, 1986, Petri, 1984a, Petri, 1984b]. Only minor differences in release of tobramycin or vancomycin were seen in human *versus* bovine grafts [Winkler, 1999]. The lower antibiotic binding capacity of cortical *versus* cancellous bone relates to differences in surface area [Winkler, 1999]. In general,

antibiotics are released from bone graft for only a short time period (hours-days) [Miclau, 1993]. In a recent study, only rifampicin demonstrated *in vitro* a release up to 21 days [Wits¿, 1999]. When human bone graft is implanted in animals to study the *in vivo* release of antibiotics, the release might be increased due to the local inflammatory response induced by the xenograft [Wits¿, 2000]. High doses of tobramycin (up to 30 mg/g) did not inhibit the osteogeneic potential of bone grafts and may therefore promote bone healing of a contaminated fracture [Lindsey, 1993, Petri, 1984a, Petri, 1984b]. Following a period of treatment with antibiotic-containing PMMA beads, Chan *et al*. reached bony union in all, and infection arrest in 95% of small infected tibial defects, by filling them with autogeneic cancellous bone graft impregnated with various antibiotics [Chan, 1998]. However, the adverse effects of therapeutic serum concentrations of antibiotics like fluoroquinolones on cartilage in fracture callus, has recently been demonstrated in an experimental model fracture healing, and should be considered in the management of (contaminated) fractures [Huddleston, 2000]. An experimental study of Thomas *et al*. suggested that the presence of demineralized bone powder itself, despite local supplemental cephalothin administration, contributed to the persistence of chronic osteomyelitis in rabbits [Thomas, 1989]. However, data on statistical significance were not provided.

I Plaster of Paris

For more than a century now, calcium sulfate, or plaster of Paris (POP) has been used as a filler of dead space in chronic osteomyelitis and bone cysts [Mackey, 1982, Peltier, 1961, Stachow, 1894]. In 1953, early after the beginning of the so-called antibiotic era, penicillin and sulfonamide added to POP showed to contribute to the healing of osteomyelitic defects in the tibia [Kovacevic, 1953]. In 1982, Mackey *et al*. demonstrated an initial high burst release of gentamicin (60-80% of the loaded antibiotic), followed by a slow *in vitro* release from POP pellets until final disintegration of the pellets at 7-9 weeks [Mackey, 1982]. To prevent high toxic serum levels, these authors suggested that it might be necessary to place these pellets, as part of their preparation, in an elutant fluid for up to 24 hours. The biodegradation (or better: resorption) of POP and the subsequent antibiotic elution period was shorter (up to 2-3 weeks) in most other *in vitro* studies [Bowyer, 1994, Dacquet,

1992, Miclau, 1993, Rauschmann, 1998, Wichelhaus, 1998]. Bowyer *et al*. suggested that antibiotic-impregnated POP beads may be indicated for short term use, such as for infection prophylaxis after acute trauma, eventually with open fracture. The optimal spectrum of antibacterial activity and the pattern of release needed for this situation might be different from the treatment of chronic osteomyelitis. Benoit *et al*. used a polylactide:coglycolide-coating to control the release of vancomycin from POP beads [Benoit, 1997]. Effective levels up to 4 weeks after implantation were measured in the rabbit s femur. In a rabbit model, the fast release of gentamicin from POP was not sufficient for the treatment of an established *S. aureus* infection [Dahners, 1987]. In two prospective clinical trials, Evrard *et al*. treated chronic osteomyelitis in 83 patients by sequestrectomy completed with insertion of POP pellets, impregnated with predominantly gentamicin or cefazolin [Evrard, 1990]. These authors reported a success rate of 61.5% and suggested that a wide extension of the lesion, which is frequent in chronic haematogenous osteomyelitis, might be a contraindication for this technique.

I PMMA cement and spacer

The mixing of antibiotics to PMMA bone cement was introduced by Buchholz and Engelbrecht in 1970 [Buchholz, 1970]. The release of methylmethacrylate monomer from polymerized bone cement lead them to investigate the release of antibiotics from PMMA. According to them, this characteristic might be beneficial when poorly vascularized tissues are encountered in implant surgery. Results of later studies supported this idea, showing that gentamicin eluted from PMMA can permeate necrotic bone, and that after systemic administration, bone concentration of tobramycin, another aminoglycoside, was dependent on vascular supply [Elson, 1977a, Winkelmann, 1978]. Four heat-resistant antibiotics were used in the experiments of Buchholz and Engelbrecht, in order to prevent inactivation due to the exothermal phase of polymerization [Buchholz, 1970]. Of these, tetracyclin was the least effective in *in vitro* experiments, probably due to its relative instability in solution. Similar results were presented by Hessert and Ruckdeschel [Hessert, 1970]. Although penicillin showed good antistaphylococcal efficacy over 6 months in the study of Buchholz, this antibiotic was not tested further because of its narrow antibacterial spectrum. Since 1969, these

authors used clinically gentamicin, erycin, or, initially, penicillin to mix with bone cement, and reported a reduction in deep infection rate of hip prostheses from 1.2 to 0.09% in the aforementioned study. Since gentamicin could not be detected in urine, these authors argued that such low systemic levels of antibiotic could not induce resistance. In a subsequent study, using 0.5 g Palacos R bone cement and in most cases no additional systemic antibiotic, Buchholz *in vivo*. reported infection rates varying from 0.9 to 2.4% for primary total hip replacements, performed from 1972 to 1975 [Buchholz, 1984]. When antibiotic-containing PMMA was used for the treatment of infected hip prostheses, the failure rate (also including loosening of implants) was higher. For the revised prostheses, the 11-years survival rate after one or more one-stage exchange procedures was 50%. The only pathogen that resulted in significantly inferior outcome in this study was *Pseudomonas*.

According to some authors, antibiotic elution from Palacos R bone cement was superior to that from other brands of cement, like Simplex P and CMW bone cement [Elson, 1977b, Hoff, 1981, Kuechle, 1991, Penner, 1999, Wahlig, 1980]. Others reported higher release of gentamicin from Simplex P or CMW bone cement *in vitro* [Bayston, 1982, Brien, 1993, Holm, 1976, Marks, 1976]. Holm and Vejlsgaard noted that sample variation, due to uneven mixing of the antibiotics with cement, lead to considerable variation in results up to 300%. In a prospective study of 49 patients undergoing a two-stage revision that involved a temporary prosthesis of antibiotic-loaded acrylic cement (PROSTALAC), Masri *et al*. did not find a statistically significant difference between Palacos-R and Simplex-P in the *in vivo* elution of vancomycin or tobramycin in joint fluid, obtained at the time of the second stage [Masri, 1998b]. Because a minimal adequate elution rate has never been demonstrated, the clinical significance of such variable differences in outcomes reported in literature, has yet to be determined [Penner, 1999].

Scepticism about the role of antibiotic-containing bone cement for infection prophylaxis came from Hill *et al* [Hill, 1977]. Prophylactic topical and systemic administration of antibiotic would be more effective in achieving bactericidal concentrations, because bone cement is not implanted at the start of the surgery.

The bacterial inhibitory efficacy of various antibiotics released from Simplex P bone cement *in vitro* was studied by Levin [Levin, 1975]. Gram-positives were much longer inhibited by clindamycin and cephalothin than by gentamicin. No significant inhibition of *P. aeruginosa* was found for any of the antibiotics tested, mixed as 3 g of powder within 40 g of PMMA powder. In a similar study, Welch demonstrated inhibition of this pathogen by gentamicin,

added in a ratio of 0.5 g or more antibiotic per 10 g PMMA [Welch, 1978].
CMW cement was also shown to be capable of releasing several antibiotics with potent antibacterial activity *in vitro*, including cephalosporins, penicillins and aminoglycosides [Ger, 1975]. Only chloramphenicol showed no inhibitory capacity in this study. In a study of Fisher *et al*. this finding was true for Simplex P, but not for CMW or Palacos [Fischer, 1977]. Of 4 cephalosporins tested, bone cement (CMW) containing cephalexin showed the longest antibacterial activity *in vitro* [Hughes, 1979].
Among the 12 antibiotics tested by Wahlig *et al*. for release from Palacos R bone cement, fusidic acid, colistin, bacitracin and dicloxacillin were released poorly *in vitro* [Wahlig, 1980]. In dogs and patients, concentrations of gentamicin were higher in connective tissue samples than in bone samples, and were detectable for 6 to 69 months in some patients.

Low quantities of release from Simplex P or Palacos R bone cement were found by Picknell *et al*. (*in vitro* and in mice or rats]. for both penicillins and other antibiotics like gentamicin [Picknell, 1977]. The activity of - according to these authors - heat-labile penicillins was not lost due to the polymerization of acrylic cements. Other authors confirmed this finding [Fischer, 1977, Hoff, 1981]
Clindamycin, nor gentamicin, was detectable in serum or soft-tissue samples after insertion of antibiotic-containing Simplex P bone cement in the rabbits thigh [Chapman, 1976].
In an experimental model of Rodeheaver *et al*., the efficacy in preventing osteomyelitis was investigated for Simplex P bone cement, containing 0.73 g of erythromycin and 0.24 g of colistin per unit of bone cement [Rodeheaver, 1983]. In their extensive study, the authors measured the highest bone concentration (respectively, 14 and 11 µg/g of bone) of these antibiotics 12 hours after implantation in the rabbit femur. The amount of bone cement inserted was not reported, but erythromycin showed a decline to undetectable levels within 16 days, more than twice as fast as that of colistin. Their data suggest that positive cultures were found in 13 to 73% of the femora treated with antibiotic-containing bone cement after inoculation with *S. aureus* or *Escherichia coli*. However, the authors defined infection as more than 10^5 colony-forming units per gram (CFU/g) of bone cultured by day 14, which definition could be supported to some extent by their histological analysis up to 28 days of a separate group of rabbits. Taking the level of 10^5 CFU/g of bone as definition of infection into account, the authors demonstrated that this bone cement was fully effective against *S. aureus*, and prevented infection in 87% of the femora inoculated with the highest dose of *E. coli*.

Antibacterial efficacy of gentamicin-containing Palacos R has been tested in a knee hemiarthroplasty model [Schurman, 1978]. Release of gentamicin was not measured in bone, but therapeutic levels were demonstrated up to 3 days in synovial fluid. Interestingly, the protective effect of gentamicin-containing Palacos R cement could not be demonstrated in rabbits challenged with *E. coli* one week after implantation. This was due to the fact that controls did not develop an *E. coli* infection, in contrast with those challenged immediately after surgery. In a preliminary study, the growth inhibitory time (*i.e.* leaching time of growth inhibitory concentration from cement in medium that is changed every day) of tobramycin was longer than that of gentamicin, using *E. coli* as the test organism [Yaniv, 1999]. These authors suggested that the two additional aminogroups of tobramycin contributed to this finding.

Several antibiotics that were not tested before to impregnate bone cement with, were studied by Beeching *et al*. for antibacterial activity *in vitro* after release [Beeching, 1986]. These included ceftriaxone, coumermycin, rifampicin, cotrimoxazole and vancomycin. Rifampicin disturbed the setting of PMMA, making it unsuitable for clinical use. Coumermycin was superior to vancomycin in inhibiting *S. aureus*, but both were ineffective against the Gram-negative organism *E. coli*.

Recently, K hn reported that most of the 18 different antibiotic-containing bone cements, available on the market at that time, contain gentamicin (as a sulphate) [K hn, 2000]. One of these, Copal, also contains clindamycin, whereas Antibiotic Simplex is nowadays available with erythromycin and colistin (AKZ), or with tobramycin. Since 1977, tobramycin-impregnated bone cement has been used for the anchorage of joint prostheses [Desoto, 1984, Duncan, 1995, Greene, 1998, Hofmann, 1995a, Kendall, 1995, Lawson, 1990, Lodenkamper, 1982, Long, 1977, Marr, 1983, Pritchett, 1992, Wininger, 1996].

In addition, several *in vitro* studies investigated this mixture as well [De Palma, 1982, Fischer, 1977, Klekamp, 1999, Yaniv, 1999]. Like Beeching *et al*., other authors (mostly American) have recently also investigated the incorporation of vancomycin in bone cement, and compared it with tobramycin, which is frequently used in non-European countries for this purpose [Wininger, 1996]. This interest reflects the emergence of methicillin resistant *S. aureus* (MRSA) in these countries [Kuechle, 1991].

Duncan and Masri reported that they measured therapeutic tobramycin and vancomycin levels in drainage fluid in patients treated for an infected prosthesis with PMMA that contained one of these antibiotics [Duncan, 1995]. Serum levels remained below 3 µg/ml in these patients, despite the addition

of as much of 3.6 g of tobramycin per 40g of bone cement. Tobramycin was shown to elute more efficiently than vancomycin in several studies [Duncan, 1995, Greene, 1998, Klekamp, 1999, Masri, 1998a, Seyral, 1994]. Lawson *et al*. reported no difference in elution characteristic from PMMA of these two antibiotics [Lawson, 1990]. The release of vancomycin from low viscosity PMMA was studied by Chohfi *et al*. in a sheep model [Chohfi, 1998]. Bone levels above the break point of susceptibility to vancomycin (4mg/l) were obtained for 6 months, although the bone levels varied considerably. For clinical use, these authors added gentamicin to the vancomycin-loaded cement, since the antibacterial spectrum of vancomycin is narrow. Yaniv *et al*. found longer leaching times from PMMA for vancomycin and a polymyxin B derivate than for gentamicin, novobiocin, and erythromycin [Yaniv, 1999]. The combination of tobramycin and vancomycin in PMMA increased the elution of one or both in the study of Penner *et al*. and Masri *et al*., whereas it had no influence on the elution of either of the two in the study of Seyral *et al*., and even decreased the elution of tobramycin in the study of Klekamp *et al* [Klekamp, 1999, Masri, 1998a, Penner, 1996, Seyral, 1994]. Variation in mixing of the bone cement, and dose or particle size of the antibiotics might explain some of these reported differences. The survival of bacteria on tobramycin- or vancomycin-containing PMMA, as noted by Kendall *et al*. in an *in vitro* model, was not seen in their subsequent prospective *in vivo* retrieval study in patients treated for an infected joint prosthesis [Kendall, 1995, Kendall, 1996]. The latter study revealed no bacterial growth and no bioadherence to bone cement that was loaded with tobramycin or vancomycin.

Risk of allergy to antibiotics in bone cement is often cited, but until now no hypersensitivity reaction has been reported, although topical use of neomycin has been reported to be associated with allergic reactions [Chapman, 1976, Chohfi, 1998, Desoto, 1984, Holm, 1976, Levin, 1975, Schurman, 1978, Wininger, 1996].

Temporary spacers, made of antibiotic-loaded PMMA, or in which some kind of endoskeleton is coated with this bone cement, like PROSTALAC, have been developed to prevent some of the drawbacks of a two-stage exchange procedure [Abendschein, 1992, Booth, 1989, Borden, 1987, Duncan, 1995, Haddad, 2000, Ivarsson, 1994, Masri, 1995, Masri, 1998c, Wilde, 1988, Younger, 1998, Younger, 1997]. Such spacers may prevent shortening of the soft-tissues and limit the loss of mobility of the joint, and provides for local antibiotic delivery during the interval between the two stages of the procedure. Articulating spacers, made from PMMA or loosely fixated with PMMA,

have been developed to allow for joint mobility during treatment [Fehring, 2000, Hofmann, 1995a, Masri, 1998b]. This modification may not improve functional outcome as compared to static spacers, but decreases the risk of bone loss [Fehring, 2000]. The elution of antibiotics from PMMA spacers is lower as compared to PMMA beads, due to the smaller surface area of spacers [Greene, 1998]. Because the load-bearing demands are lower for spacers than for bone cement used to anchor prostheses, higher amounts of antibiotics are usually loaded in these spacers. Hofmann *et al*. used 4.8 g of tobramycin with each 40-g batch of Simplex cement applied on articulating knee spacers to fill bone defects without adherence to bone [Hofmann, 1995a]. Masri *et al*. showed that the use of at least 3.6 g of tobramycin per package of bone cement to coat the temporary spacer, allows reliable antibiotic elution for at least 4 months following implantation [Masri, 1998b]. Vancomycin release was not as reliable as tobramycin in this study. Using PROSTALAC before the reimplantation of a definitive prosthesis, the infection cure rate was reported to be higher than 90% after minimum 2-year follow-up [Masri, 1998a, Younger, 1997].

The *in vivo* release of antibiotics from PMMA that is presumed to be sufficient for inhibition of local bacterial growth of most bacteria encountered in prosthesis infection, has been reported to last from a few weeks to months [Lodenkamper, 1982]. Baker and Greenham suggested that release of gentamicin from PMMA occurs from the surface of cement and through a network of bubble-like voids and cracks in its matrix [Baker, 1988]. In their study, no diffusion of gentamicin through a 0.8 mm thick PMMA disks was observed in a 9-months experiment. These findings confirmed results from a similar diffusion study by Bayston *et al* [Bayston, 1982]. Recently, Van de Belt *et al*. concluded from their *in vitro* study that the release kinetics of gentamicin from bone cement are controlled by a combination of surface roughness and porosity [van de Belt, 2000]. Masri *et al*. demonstrated that the elution of antibiotic from bone cement up to one week was merely related to surface patterns rather than changes in volume [Masri, 1995]. However, the clinical significance of this phenomenon can be questioned, since it might be more effective to improve low elution rates beyond this time frame. Kuechle *et al*. suggested the addition of dextran to PMMA beads, because the resulting increase of porosity facilitated elution of antibiotics [Kuechle, 1991]. The inclusion of such additives in PMMA was proposed earlier by Ruckdeschel *et al*., and studied by other authors [Robinson, 1989, Ruckdeschel, 1973]. Because the majority of antibiotic may remain inside the PMMA when the diffusion rate is low or zero, it is not surprising that disruption of the bone

cement mantle during revision or in experiments, can result in detectable concentration of antibiotic for months or years after insertion. Several authors confirmed such findings [Baker, 1988, Bayston, 1982, Lodenkamper, 1982, Wahlig, 1980, Wannske, 1976]. Powles *et al*. detected antibiotic concentrations even up to 10 years after insertion of antibiotic-containing bone cement [Powles, 1998].
In 1979, PMMA containing silver composites were tested by Spadaro *et al*. for antibacterial activity [Spadaro, 1979]. Of the 4 different silver composites, silver sulfate (Ag_2SO_4]. was the most effective. This cement was compared with gentamicin-containing PMMA in an experimental model of *S. aureus* osteomyelitis [Dueland, 1982]. Both cements, introduced in the rabbit tibia as prepolymerized rods, did not prevent completely the development of infection, but gentamicin-containing PMMA had a significant higher reduction of bacterial counts as compared to plain or silver-containing PMMA.
Voos *et al*. demonstrated in a goat model that tobramycin-impregnated PMMA, in the form of pin sleeves, could also protect external fixator pins from infection [Voos, 1999].

I PMMA beads

Since 1971, two years after the first clinical use of gentamicin-containing PMMA by Buchholz *et al*. for implant fixation, gentamicin-containing PMMA beads have been used for the treatment of osteomyelitis defects [Buchholz, 1981, Buchholz, 1970, Eitenmuller, 1985, Klemm, 1974, Klemm, 1979, Wahlig, 1978]. Hovelius *et al*. reported the successful use of these beads in two-stage exchange procedures [Hovelius, 1979]. Initially applied as loose beads, these beads were later attached on a stainless steel wire and placed as chains in the debrided wound cavity. This facilitated subsequent removal of the beads, usually after a period of 10-15 days. Wahlig *et al*. reported effective elution of gentamicin up to two weeks in the cortex of canine femora, in which gentamicin-containing PMMA beads were added [Wahlig, 1978]. Later, also other PMMA beads impregnated with other antibiotics were studied. Laky *et al*. showed that gentamicin and tobramycin, released from intramedullary placed PMMA beads in rabbit tibiae, were longer detectable than cephalothin [Laky, 1983]. Unfavorable elution characteristics from PMMA beads of cefazolin, ciprofloxacin and ticarcillin in canine tibiae, in

contrast to the good elution of tobramycin, vancomycin and clindamycin were reported [Adams, 1992]. In an *in vitro* study of Mader *et al*., clindamycin and tobramycin concentrations derived from PMMA beads remained much longer (through 30 weeks) above their respective breakpoint sensitivities than vancomycin (12 days) [Mader, 1997]. These and other authors compared the release of antibiotics from PMMA beads with that from other biodegradable carriers [Becker, 1994, Bowyer, 1994, Garvin, 1994, Mader, 1997]. As discussed below, a release profile from PMMA beads can be distilled from most studies that includes a lower initial burst, and a subsequent longer release of antibiotics than that of biodegradable carriers. Greene reported that the *in vitro* elution of tobramycin from PMMA beads remained above the MIC for more than 3-6 months, whereas others showed adequate tobramycin release levels eluted *in vitro* from PMMA beads for as long as 14 to 28 days [Goodell, 1986, Greene, 1998, Kirkpatrick, 1985, Miclau, 1993, Nelson, 1992, Wilson, 1988]. Nelson *et al*. studied the *in vitro* release characteristics of commercially and noncommercially prepared antibiotic PMMA beads. The addition of 2.0 g of gentamicin to hand-mixed PMMA beads did not produce any higher long term antibiotic levels than did 1.2 g of tobramycin mixed with cement of any brand [Nelson, 1992]. Others found superior elution characteristics of tobramycin- or vancomycin-loaded PMMA beads or spacers for one brand of cement (Palacos R) as compared to another (Simplex P) [Greene, 1998]. In an *in vitro* study, the release of tobramycin from a single bead over three months was calculated to be only a fraction of the theoretically available tobramycin [Goodell, 1986]. The differences in elution of tobramycin and other antibiotics from PMMA beads that are found in both *in vitro* and *in vivo* studies can be explained by the variations in size, shape, porosity and number of the beads, the amount of impregnated antibiotics and differences in sampling methods and -solutions [Adams, 1992, Bayston, 1982, Seligson, 1993, Seligson, 1985, von Frauhofer, 1985, Wilson, 1988]. For instance, elution of antibiotics from bone cement has been measured using techniques of different bioassays, scintillation counting, radioimmunoassay or a more recent technique of immunofluorescence polarization assay [Kuechle, 1991, Penner, 1999, Schurman, 1978]. To ameliorate the inhibition of bone metabolism, caused by acidification due to osteomyelitis and high local tobramycin concentration (>500 μg/ml), Murakami *et al*. suggested the incorporation of an alkalinizing agent ($Ca(OH)_2$) into PMMA beads impregnated with antibiotics such as tobramycin [Murakami, 1996, Murakami, 1997].

Treatment of a contaminated fracture with tobramycin-containing PMMA

beads in rabbits had a success rate of 75% in contrast to 25% in the group treated with tobramycin intramuscularly [Seligson, 1992]. In a similar experiment, tobramycin-containing PMMA beads were shown to be effective in preventing bacterial adherence to these beads [Lyons, 1992]. In a limited *in vivo* study of Yaniv *et al*., implantation of PMMA beads impregnated with a polymyxin B derivate was superior to gentamicin-impregnated beads in protecting rabbits against *P. aeruginosa* osteomyelitis [Yaniv, 1999]. Adequate concentrations of tobramycin were demonstrated in patients with compound fractures, who had been treated prophylactically with tobramycin-impregnated PMMA beads [Eckman, 1988]. Due randomization problems, Blaha *et al*. failed to demonstrate superiority of systemic antibiotics or gentamicin-containing PMMA beads (Septopal) [Blaha, 1993]. According to Chen *et al*., tobramycin-containing beads enhanced the effect of systemic antibiotics in bacterial clearance of wounds contaminated with *S. aureus* [Chen, 1993]. Although the authors claimed that the doses of the systemic antibiotics (tobramycin and cefazolin) produced therapeutic serum concentrations, they failed to cite the studies that could support this claim. Thus, the effect of tobramycin-containing beads might have been less pronounced when higher systemic doses were given. Successful management of chronic osteomyelitis in patients with tobramycin- or vancomycin-impregnated PMMA beads has been reported [Scott, 1988].

Antibiotic-impregnated PMMA beads have not only been studied for use in orthopaedic infections, but also for infections of the abdomen, head or chest [Alpert, 1989, Mavroudis, 1988, McKellar, 1999, Rosen, 1991]. Gentamicin-containing beads were implanted in maxillofacial wounds in dogs by Alpert *et al*., and by Rosen *et al*. in sacroperineal wounds after rectum excision in patients with rectal or anal carcinoma. McKellar *et al*. used vancomycin- and tobramycin-impregnated PMMA beads to control an infection of an implanted artificial heart assist device. In an experimental model, Mavroudis *et al*. used tobramycin-impregnated PMMA beads to treat thoracic empyema in guinea pigs

I Calcium phosphate ceramics

Hydroxyapatite ceramics (HA) and other calcium phosphates are osteoconductive materials and can be used as bone fillers. In addition, these materials

have been studied for use as a delivery system for antibiotics and other drugs. Eitenm ller *et al*. reported some experimental and clinical success in treating osteomyelitic defects in dogs and patients with flucloxacillin-, respectively gentamicin-containing HA granules [Eitenmuller, 1985]. Cornell *et al*. obtained similar results using gentamicin-containing HA beads in osteomyelitic defects in tibiae of rabbits (infection control rate 73%) [Cornell, 1993]. Cefoperazone and flomoxef were shown to be less effective released from sintered porous HA than gentamicin *in vitro*, and in subsequent experiments the latter antibiotic was detectable until approximately 2 months after insertion of this HA in rabbit tibia [Shinto, 1992]. In a subsequent study, these authors found gentamicin-HA composites to be efficacious in treating implant-related infections in rats [Korkusuz, 1993]. In a preliminary report, Itokazu *et al*. showed that porous HA blocks impregnated with different antibiotics contributed to infection control in 14 of 15 patients [Itokazu, 1998]. Although HA implants allow healing of bone defects by ingrowth of bone, reports on the biodegradability of the crystalline matrix range from full to none [Cornell, 1993, Eitenmuller, 1983, LeGeros, 1993]. Thus, theoretically, their persistence as a foreign body may complicate infection healing, when these implants are used as an alternative for bone graft to replace PMMA beads for the treatment of chronic osteomyelitic defects [Hamanashi, 1996, Korkusuz, 1993].

Self-setting HA cements loaded with antibiotics can be formed *in situ* and molded in osteomyelitic defects [Hamanashi, 1996, Otsuka, 1990, Solberg, 1999]. Higher antibiotic concentrations in such cements resulted in a longer *in vitro* release periods, but may have an inhibitory effect on bone conduction [Hamanashi, 1996, Otsuka, 1990]. Solberg *et al*. showed that both self-setting HA and PMMA, used as a gentamicin-containing void-filler for an chronic osteomyelitic defect in the rat tibia, could actually treat the infection.

Biphasic calcium phosphate ceramic, processed by compaction instead of sintering, allows for a more regular and sustained release of antibiotics, provided that the integrity of the compact can be controlled [Gautier, 2000, Trecant, 1997].

Since hydroxyapatite is brittle, self-setting apatitic glass ceramics with high compressive strength have been developed that can be loaded with antibiotics [Kawanabe, 1998, Otsuka, 1997]. The bioactivity (*i.e.* the capacity to form a chemical bond with bone) of these and other composites containing glass ceramics can enhance bone ingrowth in (osteomyelitic) bone defects [Ragel, 2000].

By changing the composition and/or porosity of the material, or the solubility

of the antibiotic agent, release rates can be varied. In addition, the technique applied for incorporating antibiotic into ceramics influence the loading capacity and release rate [Gautier, 2000]. The release rate of antibiotics from biodegradable materials like some calcium phosphate materials depends also partially on the biodegradation or bioresorption rate of these materials. This biodegradation rate is not only influenced by the chemical composition, form and cristallinity of the material, but also by variables like implantation site (osseous or non-osseous, cancellous or cortical bone), metabolic activity, presence of infection and type of cells involved [LeGeros, 1993].

Modification of the solubility of the antibiotic agents can optimize the release rate. The lower release rate of vancomycin from tricalcium phosphate ceramic beads as compared to gentamicin was explained by the higher molecular weight and lower solubility of vancomycin. The incorporation of cefotiam with a lipid before loading HA beads was shown to decrease its release rate from these beads [Yamamura, 1992]. An additional biodegradable coating has been proposed to delay the release of antibiotics from tricalcium phosphate beads [Eitenmuller, 1983]. A similar principle was applied by Radin *et al*. to prevent a burst release from an antibiotic-loaded calcium phosphate ceramic coating [Radin, 1997]. A second lipid coating on titanium discs prolonged the release of vancomycin up to 3 days in their *in vitro* study. Sasaki and Ishii included poly-L-lactic acid particles loaded with gentamicin in calcium phosphate cement implants to extend the release over a 2-months period of this antibiotic from the cement [Sasaki, 1999]. Composites of similar materials were shown earlier to release prophylactic levels of kanamycin in rabbit tibiae over a period of 3-4 weeks, and other authors sought to improve the release characteristics with additional coating formulations [Ikada, 1985, Soriano, 2000].

Because the rapid release of antibiotics from biodegradable materials may result from the adherence of antibiotics to the surface, rather than truly containment of the latter, an alternative for antibiotic-containing PMMA bone cement was developed by Gerhart *et al* [Gerhart, 1988]. Although the mechanical strength of this hydrolyzable polymer cement, with calcium -phosphate and -carbonate substitutes, is not that high as PMMA, it is more biodegradable than the latter, thereby eliminating the need for a subsequent removal of the beads. In a later study, Gerhart *et al*. could not show a difference in treatment efficacy in rats between these two types of bone cement, added with both gentamicin and vancomycin [Gerhart, 1993].

Collagen

Gentamicin-containing collagen sponges from bovine origin are commercially available since 1987. Release of the antibiotic occurs through diffusion and enzymatic degradation of the collagen [Mehta, 1996]. Becker showed that tobramycin-loaded PMMA beads delivered therapeutic concentrations longer than tobramycin loaded collagen sponge, because the first can withstand compressive loading generated by the body [Becker, 1994]. Because drug release from a collagen implant declines rapidly within one week, this material may not be optimal for the treatment of chronic osteomyelitis [Becker, 1994, Humphrey, 1998, Wachol-Drewek, 1996]. However, some authors reported clinical success, using this material as an adjunct treatment in bone and soft-tissue infections [Ipsen, 1991, Kwasny, 1994]. In addition, gentamicin-containing collagen sponge, which also has a hemostatic effect, has been used as a prophylactic agent mixed with bone graft or in cementless arthroplasty [Ascherl, 1986, Kwasny, 1994].

Biodegradable synthetic polymers

Another biodegradable carrier of antibiotic is polymerized lactic acid, in the form of polymerized dilactide or lactide/glycolide copolymer of various molecular weights [Kanellakopoulou, 2000]. The degradation rate of lactic acid polymers, and thereby the release rate of antibiotic from these implants, depends on their molecular weight [Ikada, 1985]. Lactic acid oligomer degrades faster and releases antibiotic more rapidly than high-molecular-weight polylactide [Kanellakopoulou, 1999, Wei, 1991]. Wei *et al*. showed effective local release levels of kanamycin in the vicinity of lactic acid oligomer implants in the rabbit s femur up to 4 weeks after implantation [Wei, 1991]. Release and penetration rate of this antibiotic was shown to be 10-fold higher in bone marrow than in cancellous or cortical bone. Bulk erosion of lactic acid polymers, in contrast to erosion of other polymers by layers, can result in an *in vitro* release profile of quinolones or aminoglycosides that has its maximum beyond the first week of implantation, as seen by some authors [Andreopoulos, 1996, Dounis, 1996, Wei, 1991]. Again, *in vivo* studies can show a more rapid release from these implants than *in vitro* data sug-

gest [Kanellakopoulou, 1999]. Zhang *et al*. tried to control drug release by changing the length of small antibiotic-containing PLA cylinders with an additional PLA coating, out of which the antibiotic could only diffuse through the open ends [Zhang, 1994]. These authors concluded that cefazolin and penicillin were unsuitable for sustained release longer than 25 days due to their instability in water.

Copolymers of polylactide with for instance polyglycolide, in use since 1975 as synthetic absorbable suture materials alternatively to the existing PLA and polyglycolic acid sutures, have also been studied to improve the administration of antibiotics for treatment of orthopaedic infections [Calhoun, 1997, Galandiuk, 1997, Garvin, 1994, Gilding, 1979]. In a model of a thigh muscle wound, ampicillin encapsulated in polylactide:coglycolide (PL:CG) was found to treat all rats inoculated with *Streptococcus pyogenes*, and most wounds inoculated with *S. aureus* [Setterstrom, 1985]. In addition, the efficacy of these microcapsules to prevent *S. aureus* infections was shown for tibial osteomyelitis in rabbits [Jacob, 1991]. When the treatment was delayed for one week in this model, the ampicillin-PL:CG microcapsules cleared all infections only when used in conjunction with surgical debridement. Unencapsulated ampicillin powder was less effective than its microencapsulated counterpart when applied locally [Jacob, 1991, Setterstrom, 1985]. Similar results were found in a rabbit model of prophylaxis (delayed for 2 hours) of *S. aureus* infection in tibial fractures with either cefazolin microspheres or free cefazolin powder [Jacob, 1997]. In contrast, prophylaxis with locally applied free cefazolin powder reached some moderate efficacy when this treatment started within 30 minutes after contamination with *S. aureus* [Jacob, 1993]. In the *in vitro* study of Mader *et al*., discussed above, the release of antibiotics from PMMA beads was compared with that from biodegradable materials [Mader, 1997]. Antibiotic-loaded PLA, PL:CG, and a combination of these two, did not release adequate concentrations for longer than 10 weeks and dissolved between 5 and 25 weeks. Both tobramycin and clindamycin, but not vancomycin, were released longer from PMMA. Garvin *et al*. compared the efficacy of PL:CG- and PMMA implants containing gentamicin in the treatment of *S. aureus* osteomyelitis in dogs [Garvin, 1994]. No significant difference was found in the successful treatment of this infection between the implants, both with a relatively (as compared to previous studies) high gentamicin:polymer ratio of 1:1. Calhoun and Mader used vancomycin-containing PLA- and PL:CG beads to treat *S. aureus* osteomyelitis that was induced in the rabbits two weeks earlier [Calhoun, 1997]. Although the outcome in the rabbits treated with these beads after debridement

improved as compared to controls and systemically treated rabbits, the osteomyelitis could not be eradicated completely. In another rabbit model, the local application of a PL:CG containing ofloxacin resulted in an sterilization rate of 83-91%, 4 weeks after the inoculation with *P. aeruginosa* in the distal femur [Nie, 1998]. The limited histological data presented in this study suggest that the infection continued in a higher number of rabbits than expected from the outcome of culture. Liu *et al*. studied the effect of processing parameters, like sintering temperature and compression pressure, on the *in vitro* release rate (up to 4-5 weeks) of vancomycin from PL:CG beads [Liu, 1999]. Like PLA, changes in molecular weight or composition ratio, size of beads and an additional coating also influenced the release of antibiotic from PL:CG beads [Lin, 1999c, Liu, 1999, Nie, 1995].

Du *et al*. composed soft, bioerodible implantable discs of a poly(ortho esters) polymer to release tobramycin over a 3-week period for the treatment of osteomyelitis [Du, 1997]. Results from their *in vitro* study suggested that the release rate of tobramycin could be controlled by altering the concentration of acidic additives to the implant.

Laurencin *et al*. reported that a bioerodible polyanhydride copolymer of biscarboxyphenoxypropane and sebacic acid loaded with gentamicin reduced the bacterial counts in a rat model of osteomyelitis, but 3 out of 5 infections were still present after 3 weeks of treatment [Laurencin, 1993]. Another polyanhydride (Septacin) that has been developed for the local treatment of osteomyelitis, contains erucic acid as the copolymer of sebacic acid [Stephens, 2000].

Recently, the biodegradable aminopolysaccharide chitosan was introduced as an antibiotic carrier that could be used in treatment of chronic osteomyelitis [Aimin, 1999]. In this study, the gelatinized substance, in the form of small cylinders, released gentamicin in concentrations above MIC level for *S. aureus* over 25 days *in vitro* and up to 2-8 weeks *in vivo* in rabbits. As could be noted from a small peak in the gentamicin release at 6 weeks, these implants finally cracked and burst due to swelling. *In vitro*, the inhibitory effect on the growth of *S. aureus* lasted for 7 weeks, whereas this effect ended within 3 weeks for *P. aeruginosa* [Aimin, 1999].

I Other materials and approaches

Other examples of anti-infective techniques for local application in surgery are silver ionthophoresis and the use of local drug pumps. In the first, the wound penetration of silver ions from a silver wire anode was limited to a depth of 1 cm [Becker, 1978]. The latter technique consists of drug delivery via a catheter that connects the infected site with a subcutaneously implanted or portable pump [Meani, 1994, Meani, 1998, Perry, 1992, Perry, 1985, Perry, 1986]. The risk of infection of the catheter and implantable pump has been reported by these authors, and might be less for the portable variant. Such techniques are not widely used for the local treatment of orthopaedic infections. In contrast, some antibiotic-loaded carriers are being used extensively for this purpose, and much of the ongoing research focuses on the improvement of the characteristics, like biodegradability, of these implants.

Instead of studying intrinsic biodegradation characteristics of the material, antibiotic-delivery systems might also be optimized by creating a release response that specifically depends on the local infection. Susuki *et al*. developed a hydrogel wound dressing that would not release antibiotic *per se*, but only in the presence of specific bacteria [Lin, 1999b, Suzuki, 1998]. These authors showed some inhibition of *P. aeruginosa* growth *in vitro*, by binding gentamicin to the dressing material through a peptide that is cleavable by a proteinase specific to this bacterial species.

Since not all implantations of orthopaedic prostheses and fracture-fixation hardware are associated with the use of PMMA bone cement or biodegradable pellets, alternatives for local antibiotic delivery systems have been studied for infection prophylaxis. These include the binding of antimicrobials, or proteins that inhibit bacterial adherence to the surface of an implant.

Attempts have been made to decrease bacterial adherence on orthopaedic devices by a cross-linked albumin coating. Despite *in vitro* studies that showed more than 85% inhibition of bovine or human serum albumin on bacterial adherence to titanium surfaces, only a reduction of 35% in osteomyelitis rate was reported by An *et al*., using such an implant in a rabbit model [An, 1997, An, 1996, McDowell, 1995].

Other studies aim at binding antimicrobials, either antiseptics or antibiotics, to the surface of orthopaedic devices to prevent infections. Such drugs can be attached (directly or as a coating) to the implant surface, or the existing coating (like an HA-coating of an uncemented prosthesis) can be loaded with antimicrobials. These techniques have also found their application in other

fields of surgery for the prevention of infections, related to prosthetic-devices like urinary catheters, cerebrospinal fluid shunts, vascular grafts, and pacemakers [Kockro, 2000, Leblebicioglu, 1999, Raad, 1995, van Wachem, 1998]. In 1994, Dunn *et al*. introduced a technique to attach ciprofloxacin electrostatically to the surface of a porous coated titanium-6% aluminium-4% vanadium alloy (Ti-6Al-4V) implant [Dunn, 1994]. He showed that more antibiotic (up to 3 mg/cm^2, released *in vitro* over a period of 5 days) could be attached to the implants using this technique, as compared to a previously reported method that involved the electrostatically attachment of gentamicin to the anodized surface of Ti-6Al-4V [Dunn, 1993]. In their latest study, the authors did not perform fatigue tests, but earlier results showed that a microporous oxide coating decreased the fatigue strength of Ti-6Al-4V samples with 19% [Dunn, 1993, Dunn, 1994]. Price *et al*. impregnated gentamicin in a PLGA-film on a stainless steel fracture plate [Price, 1996]. The *in vitro* release rate of gentamicin as a function of antibiotic concentration of the film had its optimum at a 20% loading concentration of the coating. The coating with a higher load did not maintain effective levels in elution through 20 days, despite an initially high release rate (burst effect). Their *in vitro* bacterial inhibition study, using the implant containing 40 mg of gentamicin (20% loading concentration), showed an almost complete reduction of bacterial growth over 24 days.

In an experimental study of Darouiche *et al*., the use of intramedullary nails, coated with an antiseptic combination of chlorhexidine and chloroxylenol, for fixation of tibial fractures was associated with a significant reduction in the rate of device-related osteomyelitis (relative risk, 6.81) [Darouiche, 1998]. This reduction might have been higher when the rabbits would not have received cefazolin as an additional perioperative infection prophylaxis. Campbell *et al*. incorporated chlorhexidine into the hydroxyapatite coating of external fixator pins [Campbell, 2000]. A lipid overlayer was needed to retard the initial release rate to over 24 hours. Improvement of release characteristics seems warranted, because the indication for use of this type of coating is the prevention of infection of external fixator pins, which, as the authors indicated, may remain in place for several months. In other studies of pin tract infections, silver-coated pins were used for this indication. *In vitro*, some reduction in bacterial adherence was seen using these pins [Wassall, 1997]. Only a small reduction in infection rate was found in a goat model, whereas Masse *et al*. found no significant reduction in a prospective randomized clinical study [Collinge, 1994, Masse, 2000]. Because the implantation of silver-coated screws in 24 patients with lower limb fractures also resulted

in a significant increase in serum iron levels, the authors of the latter study considered it ethically unacceptable to continue their investigation.

I Summary remarks on antimicrobial-loaded implants

In this chapter, the limited review of literature on antimicrobial-loaded carriers that have been proposed for use in musculoskeletal infection shows that treatment options are evolving. The concept of a local anti-infective agent that is directed specifically at the local site of the infectious process has stood firmly for more than half a century now in the management of orthopaedic infections. Nevertheless, in the latest decades many new variants of antimicrobial-loaded carriers have been studied, aiming at a still more efficacious antimicrobial treatment. Antibiotic-containing bone cement, like the one that is the subject of experimental studies described in the next chapters of this thesis, is most often used in the management of prosthesis infection. In bead form, this cement is used frequently to treat chronic osteomyelitic processes or in exchange procedures for an infected implant. Other carriers are primarily indicated for infection prophylaxis in fracture fixation, or should eventually be fully replaced by ingrowth of surrounding bone or soft tissues in large osteomyelitic defects. Thus, the site and extent of an infectious process directs the choice for a specific type of antimicrobial-loaded implant. However, because many new materials have become available, which allow tailoring of release and biodegradation characteristics of such implants, the armamantarium of local anti-infective treatment has grown.

Table 1

Material	Form	Antimicrobial	Characteristic	Reference
Bone graft				
Bone graft	Morselized	Penicillin	Biodegradable	[de Grood, 1947].
Bone allograft	Morselized	Tobramycin	Biodegradable	[Petri, 1984b]
		Cephalothin		
Bone autograft	Morselized	Ciprofloxacin	Biodegradable	[Durig, 1986]
Bone allograft	Morselized	Tobramycin	Biodegradable	[McLaren, 1986]
Bone allograft	Morselized	Tobramycin vancomycin	Biodegradable	[McLaren, 1988]
Bone allograft	Morselized	Cephalothin	Biodegradable	[Chan, 1998]
		Vancomycin		
		Piperacillin		
		Ticarcillin		
Bone allograft	Morselized	Penicillin	Biodegradable	[Witsø, 1999]
		Dicloxacillin		
		Cephalothin		
		Netilmicin		
		Vancomycin		
		Ciprofloxacin		
		Clindamycin		
		Rifampicin		

Plaster of Paris				
Calcium sulfate	Cylinder	Penicillin Sulfonamide	Biodegradable	[Kovacevic, 1953]
Calcium sulfate	Pellet	Gentamicin Fucidin Cefazolin Lincomycin	Biodegradable	[Mackey, 1982]
Calcium sulfate	Paste	Gentamicin	Biodegradable	[Dahners, 1987]
Calcium sulfate	Bead	Teicoplanin	Biodegradable	[Dacquet, 1992]
Calcium sulfate	Cylinder	Tobramycin	Biodegradable	[Miclau, 1993]
Calcium sulfate	Pellet	Gentamicin + Vancomycin	Biodegradable	[Rauschmann, 1998]
PMMA cement and spacer				
PMMA	Cement plug	Gentamicin Penicillin (Tetracycline)	Erythromycin	[Buchholz, 1970]
PMMA	Cement	Gentamicin Penicillin Tetracycline		[Hessert, 1970]
PMMA	Cement disk	Methicillin Carbenicillin		[Levin, 1975]

Cephalothin	Cement plug	PMMA	[Marks, 1976]
Neomycin			
Kanamycin			
Colistin			
Polymyxin B			
Erythromycin			
Lincomycin			
Clindamycin			
(Tetracycline)			
Oxacillin	Cement plug (= disk)	PMMA	[Picknell, 1977]
Cefazolin			
Gentamicin			
Ampicillin			
Amoxycillin			
Cloxacillin			
Flucloxacillin			
Methicillin			
Carbanicillin			
Ticarcillin			
Cephaloridine			
Gentamicin			
Fusidic acid			
Clindamicin			

PMMA	Disk/bar	Ampicillin	[Fischer, 1977]
		Cephalosporin	
		Chloramphenicol	
		Colistin	
		Gentamicin	
		Kanamycin	
		Lincomycin	
		Penicillin	
		Polymyxin	
		Carbenicillin	
		Rifamycin	
		Streptomycin	
		Tetracyclin	
		Tobramycin	
PMMA	Pellet	Tetracyline	[Ger, 1975]
		Ampicillin	
		Cephalothin	
		(Chloramphenicol)	
		Cloxacillin	
		Fusidic acid	
		Gentamicin	
		Kanamycin	
		Neomycin	
		Lincomycin	

PMMA	Cement	Oxacillin		[Long, 1977]	
		Cefazolin			
		Tobramycin			
		Erythromycin			
PMMA	Cement	Cefuroxime		[Hughes, 1979]	
	Cephalexin				
		Cephaloridine			
		Cephalothin			
PMMA	Rod	Silver		[Spadaro, 1979]	
PMMA	Cement	Tobramycin		[Marr, 1983]	
PMMA	Cement	Vancomycin +	Low viscous	[Cholfi, 1998]	
		Gentamicin			
PMMA	Cement	Cephalothin		[Beeching, 1986]	
		Coumermycin			
		Fusidic acid			
		(Ceftriaxone)			
		Cotrimoxazole			
		(Rifampicin)			
		Vancomycin			
		Gentamicin			
PMMA	Disk	Daptomycin	Amikacin	[Kuechle, 1991]	
		Vancomycin			
PMMA	Spacer	Tobramycin		[Booth, 1989]	
PMMA	Sleeve	Tobramycin		[Voos, 1999]	

PMMA beads

PMMA	Bead	Gentamicin		[Klemm, 1974]
PMMA	Bead	Gentamicin Tobramycin	Cephalothin	[Laky, 1983]
PMMA	Bead	Vancomycin	Tobramycin	[Scott, 1988]
PMMA	Bead	(Cefazolin) (Ciprofloxacin) (Ticarcillin) Clindamycin Tobramycin Vancomycin		[Adams, 1992]
PMMA + calcium hydroxide	Bead	Tobramycin	PH regulation	[Murakami, 1997]
PMMA	Bead	Novobiocin Polymyxin B Gentamicin Erythromycin Cefazolin Vancomycin		[Yaniv, 1999]

Calcium phosphate ceramics

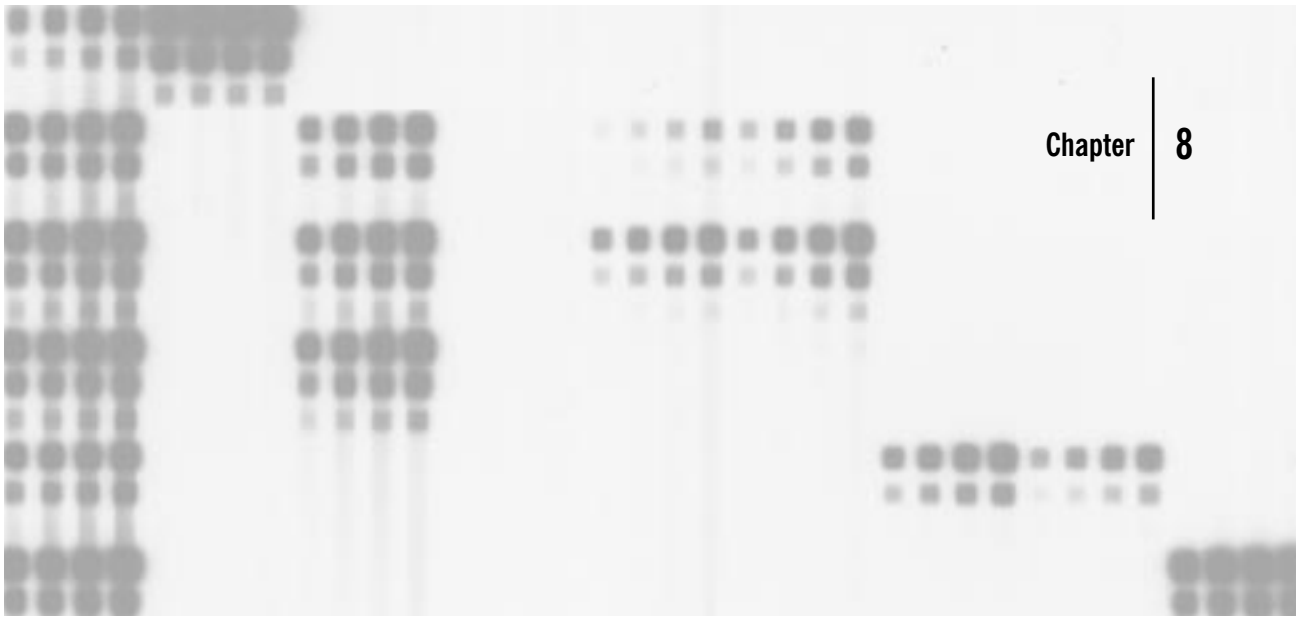
Tricalcium phosphate +copolymer coating	Granule	Flucloxacillin phosphomycin	Osteoconductive	[Eitenmuller, 1983]
Hydroxyapatite	Granule	Flucloxacillin Phosphomycin Povidone-iodine Gentamicin	Osteoconductive	[Eitenmuller, 1985]
Hydroxyapatite (TeCP-DCP)	Cement pellet	Cephalexin Norfloxacin	Osteoconductive Self-setting	[Otsuka, 1990]
Hydroxyapatite	Block	Gentamicin Cefoperazone Flomoxef	Osteoconductive	[Shinto, 1992]
Hydroxyapatite/lipid	Bead	Cefotiam	Osteoconductive	[Yamamura, 1992]
Hydroxyapatite + Poly(D,L-lactic acid)+	Cylinder	Kanamycin	Biodegradable	[Ikada, 1985]
Hydroxyapatite (TeCP-DCP)	Cement cylinder	Vancomycin	Biodegradable Self setting	[Hamanashi, 1996]
Tricalcium phosphate	Sphere	Gentamicin Vancomycin	Osteoconductive	[Brouard, 1997]
Hydroxyapatite/tricalciumphosphate (biphasic)	cylinder	Vancomycin	Osteoconductive	[Trecant, 1997]
Oxyfluorapatite-wollastonite glass ceramic + acrylic resin	Cement pellet	Cephalexin	Osteoconductive, Self-setting	[Otsuka, 1997]
Oxyfluorapatite-wollastonite glass ceramic	Block	Isepamicin	Osteoconductive	[Kawanabe, 1998]

Hydroxyapatite	Block	Cefmetazole Isepamicin Vancomycin Cefazolin Arbekacin	Osteoconductive	[Itokazu, 1998]
Hydroxyapatite (TeCP-DCP)	Cement	Gentamicin	Biodegradable Self setting	[Solberg, 1999]
Hydroxyapatite + Poly(D,L-lactic acid)+	Cylinder	Gentamicin	Biodegradable	[Sasaki, 1999]
Collagen				
Collagen	Sponge	Gentamicin	Biodegradable	[Ascherl, 1986]
Collagen	Sponge	Tobramycin	Biodegradable	[Becker, 1994]
Synthetic biodegradable polymers				
Poly (D,L-lactide):co-glycolide	Microsphere	Ampicillin	Biodegradable	[Setterstrom, 1985]
Poly(D,L-lactic acid)	Rod	Kanamyin B	Biodegradable	[Wei, 1991]
Poly (D,L-lactide):co-glycolide	Microsphere	Ampicillin	Biodegradable	[Jacob, 1991]
Poly(L-lactid acid)	cylinder	Ciprofloxacin	Biodegradable	[Teupe, 1992]
Polyglycolic acid and poly(L-lactic acid)	Cylinder	Ciprofloxacin	Biodegradable	[Winckler, 1992]
Poly (D,L-lactide):co-glycolide	Microsphere	Cefazolin	Biodegradable	[Jacob, 1993]
Poly(D,L-lactide)	Cylinder	Gentamicin	Biodegradable	[Zhang, 1994]

		Cefazolin		
		Penicillin		
Poly (D,L-lactide):co-glycolide	Implant	Gentamicin	Biodegradable	[Garvin, 1994]
Poly (D,L-lactide):co-glycolide	Bead	Ofloxacin	Biodegradable	[Nie, 1995]
Poly(lactid acid)	Slab	Ciprofloxacin	Biodegradable	[Andreopoulos, 1996]
		Ofloxacin		
Poly(D,L-lactide):co-glycolide + polylactic acid	Bead	Clindamycin	Biodegradable	[Mader, 1997]
		Tobramycin		
		Vancomycin		
PL:CG	Bead	Minocycline	Biodegradable	[Galandiuk, 1997]
		Amikacin		
Poly(D,L-lactic acid)	Slab	Ciprofloxacin	Biodegradable	[Kanellakopoulou, 1999]
		Perfloxacin		
Poly(ortho esthers)	Disk	Tobramycin	Biodegradable	[Du, 1997]
Poly(propylene fumarate)-methylmethacrylate	Cylinder	Gentamicin Vancomycin	Biodegradable	[Gerhart, 1988]
Polyanhydrides	Rod 3*5	Gentamicin	Biodegradable	[Laurencin, 1993]
Chitosan -aminopolysaccharide	Cylinder	Gentamicin	Biodegradable	[Aimin, 1999]
polyhydroxybutyrate-co-hydroxyvalerate	Rod	Sulfabactam Cefoperazone	Biodegradable	[Yagmurlu, 1999]
Other materials and approaches				
Silver	Wire	Silver ion	Iontophoresis	[Becker, 1978]
Drug pump		Amikacin	Implantable	[Perry, 1986]

Drug pump		Vancomycin Amikacin	Portable	[Meani, 1994]
Coating (surfactant)	Porous implant	Oxycillin	Cationic surfactant	[Manley, 1988]
Oxide layer	Titanium implant	Gentamicin		[Dunn, 1993]
Coating	Stainless steel IM nail	Chlorhexidine Chloroxylenol		[Darouiche, 1998]
Coating	Stainless steel pin	(Silver)		[Nelson, 1999]
		Chlorhexidine + chloroxylenol		
Calcium phosphate ceramic/lipid	Coating on titanium disc	Vancomycin	Osteoconductive	[Radin, 1997]
Coating (Polylactide-co-glycolide)	Stainless steel plate	Gentamicin	Biodegradable	[Price, 1996]
Polyvinyl alcohol hydrogel	Dressing	Gentamicin	Enzyme-specific release	[Suzuki, 1998]
Calcium alginate	Dressing	Vancomycin	Biodegradable	[Lin, 1999b]
Fibrin	Sealant	Cefotaxim	Biodegradable	[Zilch, 1986]
Glass/PMMA/poly(lactid acid)	Platelet	Gentamicin	Bioactive	[Ragel, 2000]

Release of tobramycin from tobramycin-containing bone cement in bone and serum of rabbits



Chapter 8

I Introduction

Since 1969, various combinations of antibiotics and bone cement have been used in the prevention and treatment of arthroplasty infections [Buchholz, 1970, Elson, 1977b, Murray, 1984]. Tobramycin, like other aminoglycosides, is heat-stable, which makes it suitable for incorporation in polymethylmethacrylate (PMMA). In addition, tobramycin is potentially active against the most frequently found causative infective organisms in arthroplasty surgery (Staphylococcus species and aerobic Gram-negative bacilli).

In order to be effective in preventing or treating arthroplasty infections antibiotic-containing bone cement must fulfil two requirements. Firstly, the antibiotic should elute in a concentration that exceeds the susceptibility level for the infecting bacteria. This is the minimal inhibitory concentration (MIC), defined as the lowest concentration of antibiotic that prevents visible growth after an 18-24 hours incubation *in vitro*. Secondly, this concentration should be reached at the site of contamination or infection. Thus in arthroplasty surgery, the antibiotic concentration should be sufficiently high (i.e. well above the MIC) at the site of the implant, especially at the bone-cement interface and in the surrounding bone. In addition, the antibiotic concentration in serum should not exceed toxic levels.

Release studies of tobramycin-containing bone cement are limited. Seyral *et al*., Miclau *et al*. and Lawson *et al*. demonstrated *in vitro* elution of tobramycin from PMMA bone cement [Lawson, 1990, Miclau, 1993, Seyral, 1994]. The presence of tobramycin in wound drainage, serum and urine of patients who underwent total hip arthroplasty with tobramycin-containing cement has been shown [Brien, 1993, Pritchett, 1992]. In addition, tobramycin release from cement beads and spacers has been studied *in vivo* [Adams, 1992, Masri, 1995, Seligson, 1992].

In the present study, we determined the release of tobramycin from tobramycin-containing bone cement in an animal model. Tobramycin release in blood and bone as a function of time was measured in rabbits, after insertion of tobramycin-containing cement into the femur. The longest follow-up period was 28 days.

I Materials and methods

Animals

Thirty-nine healthy adult female New Zealand White rabbits (Ico:NZW, Broekman Instituut BV, Someren, The Netherlands), ranging in weight from 2.8 to 3.5 kg, were obtained one week before surgery to acclimatize to the housing in the Central Animal Laboratory Institute, Utrecht University. The animals were caged individually, fed with 80-100 g antibiotics-free rabbit diet (Hope Farms Standard laboratory Diet LKK-20, Hope Farms BV, Woerden, The Netherlands) and water *ad libitum*.

Cement

Surgical Simplex P bone cement, containing 1.0 g tobramycin as a sulphate in 60.0 g methyl metacrylate (batch: # N6043), was supplied by Stryker-Howmedica-Osteonics, Rutherford, NJ.
This radiopaque bone cement is a mixture of a liquid component (monomer) and a powder component (polymer), both sterilized. The composition of the powder component is: polymethyl methacrylate (6.0 g), methyl methacrylate-styrene copolymer (30.0 g) barium sulphate (4.0 g) tobramycin (as a sulphate, 1.0 g). The liquid component (20 ml) is composed of methyl methacrylate (97.4% vol/vol), N,N-dimethyl-p-toluidine (2.6% vol/vol) and hydroquinone (75 – 15 ppm). The bone cement (4¡C) was vacuum-mixed (-0.9 bar) for 1 minute on the surgical table (Simplex cement vacuum-mixer; Stryker-Howmedica-Osteonics, Rutherford, NJ).

Experimental design

Tobramycin-containing bone cement was introduced into the medullary canal of the right femur of the rabbits. At 1, 3, 7, 14, 21 and 28 days, groups of six rabbits were killed and the tobramycin levels in bone adjacent to the cement were assayed. Bone from the left femur without cement served as control. Tobramycin serum levels were also measured.
The experimental design was approved by the institutional review board (Animal Care and Use Committee, Faculty of Medicine, Utrecht University).

Anesthesia

Surgery was performed under general inhalation anesthesia. The anesthesia was prepared by an intramuscular (i.m.) injection of 0.2 mg methadone (methadoneHCl 10 mg/ml), 0.2 mg acepromazinemaleate (Vetranquil;

Sanofi Sant BV, Maassluis, The Netherlands) and 0.5 mg atropine (Atropine-sulphate, Kobivet, Etten-Leur, The Netherlands). A pressure line was introduced into the auricular artery for measuring blood pressure. Subsequently, anesthesia was induced by an intravenous injection of 8-12 mg etomidate (Hypnomidate; Janssen Pharmaceutica BV, Tilburg, The Netherlands). An endotracheal tube (#3) was introduced through which the anesthesia was maintained with a 1:1 mixture of nitrous oxide, oxygen and halothane 1% (Albic BV, Maassluis, The Netherlands). If the blood pressure fell after insertion of the cement, dopamineNaCl (0.25 mg/ml; ICN Pharmaceuticals Holland BV, Zoetermeer, The Netherlands) was injected intravenously.

Operative technique

Surgery was performed under strict aseptic conditions. The skin of the outer right thigh was clipped and the rabbit was placed with its left side on the table. The operative area was disinfected with a 2% tincture of iodine and isolated by sterile drapes. Subsequently, a skin incision (approximately 3 cm) was made over the trochanter tertius of the right femur, parallel to the femur shaft. The trochanter tertius was exposed by splitting the fascia, retracting the femoral biceps and coccygeofemoral muscles posterio-medially, and scraping the periost. Using an air-pressured AO minidrill the cortex was penetrated by a small drill (diameter 1.2 mm). Subsequently this hole was widened and the femoral canal was reamed with drills and fraises up to 4.0 mm in width until a silicon tube (monitor line, outer diameter 3.0 mm) could be inserted. After vacuum mixing of the PMMA-tobramycin bone cement, a small 6-ml syringe, with a 2-cm long silicon tube (outer diameter 3.0 mm, inner diameter 1.5 mm) attached to it, was filled with cement. The syringe was weighed on an electronic balance (PT150; Sartorius-Instrumenten BV, Nieuwegein, The Netherlands).

Before insertion of cement, the medullary canal was washed with sterile physiologic saline and suctioned. The syringe was placed in an adapted device on an applicator gun and approximately 1.2 ml cement was injected gently into the femoral canal, while the syringe was slowly being retracted. Subsequently, the syringe was weighed again in order to know the exact amount of cement injected. After polymerization of the cement and wound drainage with sterile saline solution, the fascia, subcutis and cutis were closed with Vicryl 3-0 (Ethicon GmbH & Co KG, Norderstedt, Germany). Pain relief was provided by 0.3 ml nalbufine (Nubain; Lamepro BV, Raamsdonkveer, The Netherlands) i.m. immediately postoperative, and subsequently 0.3 ml buprenorfine (Temgesic; Rechitt and Colman Products,

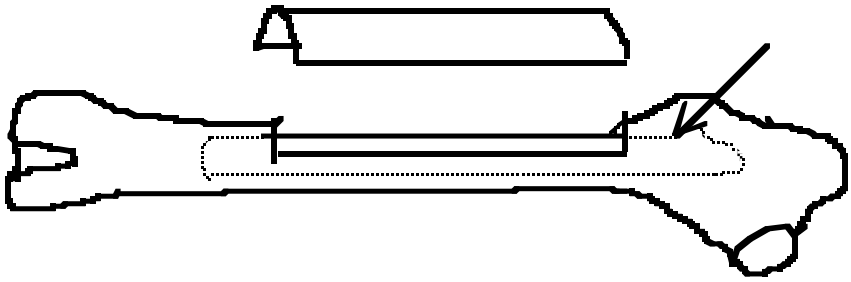


Figure 1. Femur of rabbit with lateral half of cortex excised. Cement (dotted line) was injected proximally (arrow).

Kingston-upon-Hull, United Kingdom) i.m. (if necessary, buprenorfine injection was repeated postoperatively).

Follow-up

Postoperatively, routine AP and lateral x-rays of the right femur visualized the position of the cement. The rabbits recovered in a temperature-controlled recovery cage. The rabbits were monitored by a daily clinical examination, with special attention to wound healing, the presence of a fracture, eating, activity level and body temperature. At 1, 3, 7, 14, 21 and 28 days, groups of six rabbits were killed with an overdose of pentobarbital N_2 (Euthesate; Apharmo BV, Arnhem, The Netherlands) intravenously.

Tobramycin assay technique

Tobramycin concentrations in bone and serum were measured by fluorescence polarization immunoassay (TDX, Abbot Laboratories, Chicago, IL) The minimal detectable tobramycin concentration was 1.0 μg/g in bone and 0.10 μg/ml in serum.

Bone

The left (control) and right femur from all animals were excised and cleaned of tissue debris. First a bone fragment was taken from the left femur from a region corresponding to the right femur. Secondly, using a high-speed dental drill with a circular metal saw, the external surface of the right femur was notched circumferentially at each end of the shaft and longitudinally on two sides, posterior and anterior. An osteotome was used to free the lateral half of the bone from the medial half, with the cement core *in situ* (Figure 1). Care

was taken not to damage the cement. Only full-thickness cortex adjacent to the cement was used. The bone fragments of the lateral half of each femur were ground in a metal mortar. Samples of 1.00 g of bone were homogenized in 10.0 ml Phosphate Buffered Saline (PBS, pH 7.4) using a Polytron PT 3100 tissue grinder (Kinetica Benelux BV, Best, The Netherlands), 3 minutes at 2500 rpm and 5 minutes at 6000 rpm. The homogenate was stored at 4¡C overnight. Following centrifugation (3000 rpm, 7 minutes, 4¡C) the supernatant was removed for tobramycin TDX assay.

Serum

Blood samples were taken from the auricular vein of all rabbits on the day of killing. The 28-days rabbits also had tobramycin serum levels measured preoperatively, subsequently at 12 hours, and at 1, 3, 7, 14 and 21 days postoperatively. These samples were centrifuged at 3000 rpm for 9 minutes at 4¡C, after which the serum was collected for tobramycin TDX assay.

E-test

The minimal inhibitory concentration (MIC) of tobramycin against *S. aureus* Wood 46 was determined using the E-test (E test; AB Biodisk, Solna, Sweden) [Brown, 1991].

Statistical analysis

Dixon s Q-test was used to determine outliers in extreme values of tobramycin concentration.

I Results

The mean weight (– standard error of the mean, SE) of cement inserted was 1.24–0.03 g.

Two rabbits died during surgery. Histology showed fat and bone marrow embolisms in lungs and heart. All other animals recovered well. Post-operative x-rays showed no fractures or cement outside the femur. A fracture was seen at necropsy in one rabbit at day 21 (excluded from study).

Figure 2 shows the tobramycin concentration (mean–SE) for a period of up to 4 weeks in serum and bone of both the operated right femur and the left femur (control). Extreme values in each follow up group of rabbits were not

outliers (Dixon s Q test, $p<0.05$). Peak tobramycin concentrations in the right and left femur were reached on day 3 (29.9–7.0 vs. 1.7–0.3 μg/g bone respectively, mean–SE).
The tobramycin concentration in the right femur decreased to 5.10–2.2 μg/g (mean–SE) over 28 days. The tobramycin concentrations in serum remained constant throughout the study, just above the detection limit of 0.1 μg/ml (highest concentration at 12 hours 0.25–0.0 μg/ml, mean–SE).
The minimal inhibitory concentration of tobramycin for *S. aureus* Wood 46 was determined as 0.125 μg/ml.

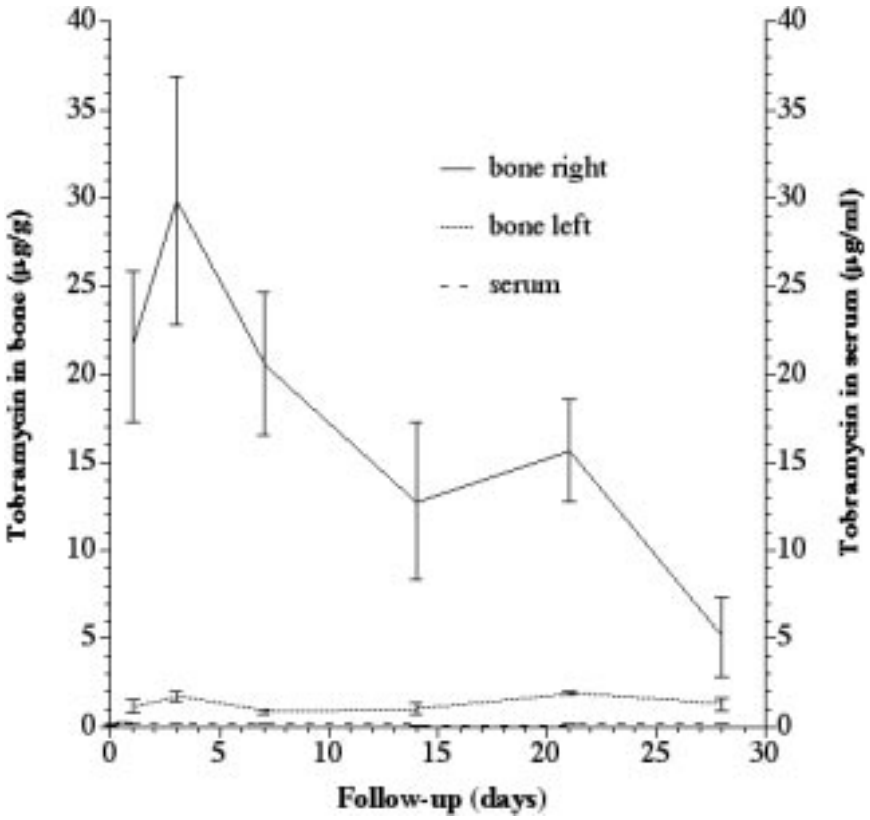


Figure 2. Tobramycin release (mean–SE) in bone of right femur (insertion of cement) and left femur (control), and in serum.

I Discussion

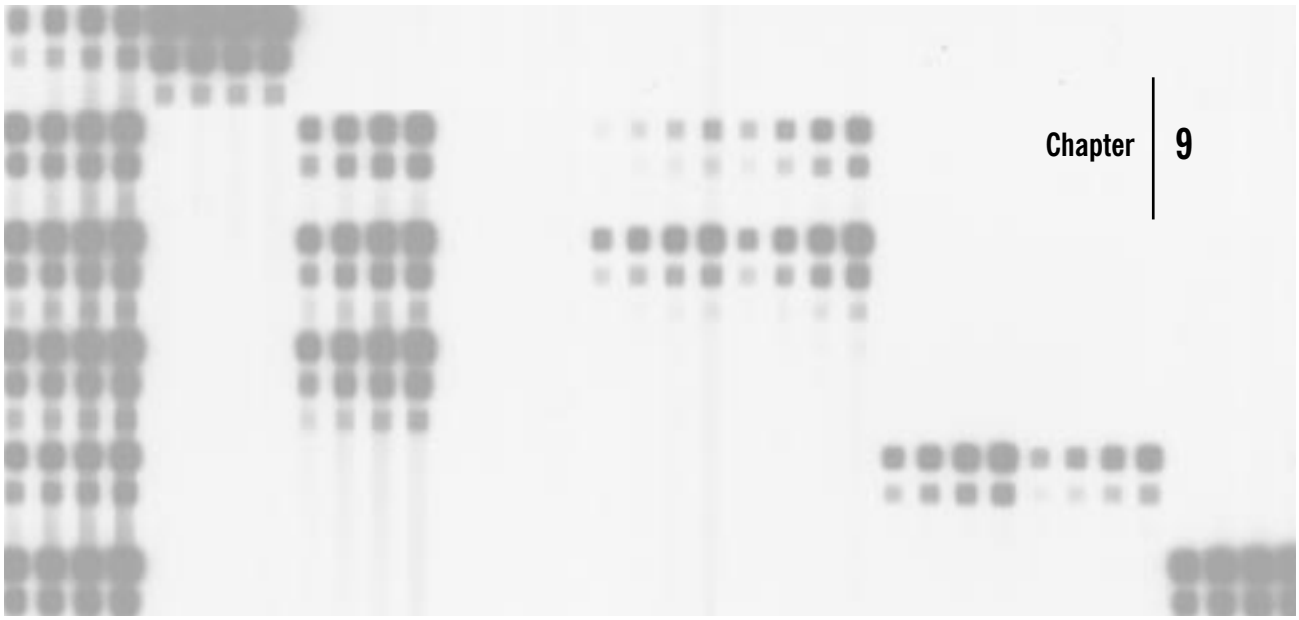
In the present study, tobramycin concentrations in the rabbit femoral cortex adjacent to the inserted bone cement exceeded the MIC for *S. aureus* Wood 46 for up to 28 days: On day 3 (peak level), a mean tobramycin concentration of 239 times MIC was measured, and on day 28 still 40.8 times MIC. These results, showing local antibiotic concentrations above the MIC levels of frequently encountered bacteria in arthroplasty infection, suggest some efficacy in the prevention and/or treatment of such an infection. Furthermore, the measured tobramycin concentrations in bone appear to be low enough to prevent bone toxicity: In recent studies, local bone concentrations of tobramycin below 200-500 µg/ml are recommended [Miclau, 1995, Murakami, 1996]. The threshold for tobramycin-induced nephrotoxicity, another side effect associated with aminoglycosides, is 6.0 µg/ml [Seligson, 1992]. Only serum levels of tobramycin well below this threshold could be detected.

The exact mechanism of antibiotic release from PMMA-bone cement is not yet clear. Amongst others, elution characteristics of antibiotic-containing bone cement depend on amount of antibiotic, and the surface area-to-volume ratio of the cement and the presence of voids and cracks in the cement [Baker, 1988, Gerhart, 1988, Marks, 1976, Masri, 1995, Miclau, 1995, Schurman, 1978, Seyral, 1994, Welch, 1978, Wroblewski, 1977]. Therefore, the release pattern of a certain formulation of antibiotic-containing bone cement is difficult to predict exactly from earlier studies, especially from those using different types of antibiotics and/or bone cement. An efficacy study on such a new formulation to be used in arthroplasty surgery should be preceded by a release study *in vivo*.

Because the size of the rabbit femur is relatively small as compared to human bone, the risk of creating a rise in intramedullary pressure by entrapment of air during insertion of cement is present. This rise in intramedullary pressure is a major pathogenic factor for the development of fat embolism syndrome [Hofmann, 1995b]. Not surprisingly, histology showed embolisms to be the cause of death of two rabbits who died just after insertion of the cement.

In conclusion, after insertion of tobramycin-containing bone cement in the femur of a rabbit, the tobramycin release in bone up to 28 days results in high concentrations next to the cement. Serum concentrations of tobramycin are well below systemic toxicity threshold. Subsequent studies were performed in order to evaluate the prevention of actual implant infection using this premixed tobramycin-containing bone cement.

Prophylaxis of implant-related staphylococcal infections using tobramycin-containing bone cement



Chapter 9

I Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are the most frequently found organisms involved in arthroplasty-related infection. Incidence rates after total joint arthroplasty of *S. epidermidis* (26-38% of infections) are somewhat higher than those for *S. aureus* (16-24% of infections) and have increased in the last decade [Fitzgerald, 1994, Garvin, 1993, Ostendorf, 2001, Sanzen, 1988, Tsukayama, 1996] Apparently, staphylococci have surface properties that enable them to adhere and grow on implant-surfaces. Some of these surface factors that mediate adhesion to biomaterials have been more or less well characterized for *S. epidermidis* [Fleer, 1990] This surface-adherent growth mode might even hamper killing of the bacteria by antibiotics [Naylor, 1990]. Darouiche *et al*. showed that *S. epidermidis*, grown on stainless steel nuts in presence of vancomycin, could not be eradicated completely even though high levels of vancomycin were reached in the biofilm [Darouiche, 1994].

The use of antibiotic containing bone cement in arthroplasty surgery provides for high concentrations of antibiotics at the implant side, whereas the low systemic levels of antibiotics reduce possible systemic toxicity such as in case of aminoglycosides. Among the latter class of antibiotics, tobramycin is one of the prime candidates for the preparation of antibiotic bone cement, because of its antibacterial spectrum. This spectrum includes staphylococci as well as aerobic Gram-negative bacteria like *Pseudomonas aeruginosa*, which is an example of another isolate involved in arthroplasty-related infections [Buchholz, 1981, Fitzgerald, 1994]. As compared to gentamicin, tobramycin has a greater activity against *Pseudomonas aeruginosa* [Edson, 1991, Lode, 1998, Whelton, 1984]. In numerous hospitals, especially in the United States, antibiotic containing bone cement is prepared by adding tobramycin powder perioperatively to bone cement and subsequent hand-mixing [Heck, 1995]. This hand mixing of antibiotics with bone cement does not allow for a consistent and controllable quality of the cement. A standardized preparation of antibiotic-containing bone cement benefits not only future infection-based efficacy studies, but also the evaluation of its biomechanical properties. Although adding low quantities (approximately 1 g per unit) of tobramycin has minimal effects on the fatigue life of cement, amounts of more than 2 g of antibiotics per unit of cement has been shown to decrease the strength of cement [Davies, 1991, Lautenschlager, 1976].

The efficacy of premixed tobramycin-containing bone cement in preventing

implant infections has not been investigated before. In a previous study on the release of tobramycin from commercially prepared tobramycin-containing bone cement, the minimal inhibitory concentrations of this antibiotic against staphylococci were exceeded for up to 28 days [Nijhof, 1997]. The purpose of the present study was to investigate the efficacy of the same bone cement in the prevention of local *S. aureus* and *S. epidermidis* infections.

Materials and Methods

Experimental design

Premixed tobramycin-containing bone cement or plain bone cement (controls) was introduced into the medullary canal of the rabbits' right femur. Prior to cement injection, this medullary canal had been inoculated with *S. aureus* or *S. epidermidis* in five increasing doses. To monitor infection, the amount of bacteria in the cortex of the femur was quantified after 7 and 28 days, when the animals were killed. In both bacteria groups 40 rabbits (20 tobramycin-cement, 20 controls) were killed after 7 days, and 20 rabbits (10 tobramycin-cement, 10 controls) after 28 days.
The guidelines according to the Dutch act on animal experiments (1985) have been observed.

Animals

120 Healthy adult female New Zealand White rabbits (Ico:NZW, mean weight 3.1 kg, range 2.6 to 3.6 kg) were obtained one week prior to surgery to acclimatize them to the housing in the Central Animal Laboratory Institute, Utrecht University. The animals were fed with 80-100 g antibiotics-free rabbit diet and water *ad libitum*.

Cement

Surgical Simplex P bone cement, premixed with 1.0 g of tobramycin as a sulphate in 60 g methyl methacrylate (batch: N6043), was supplied by Stryker-Howmedica-Osteonics, Rutherford, NJ). This radiopaque bone cement is a mixture of a liquid component (monomer) and a powder component (polymer), both sterilized. The composition of the powder component is: polymethyl methacrylate (6.0 g), methyl methacrylate-styrene copolymer (30.0 g), barium sulphate (4.0 g) and tobramycin (as a sulphate, 1.0 g). The liquid

component (20 ml) is composed of methyl methacrylate (97.4% vol/vol), N,N-dimethyl-p-toluidine (2.6% vol/vol) and hydroquinone (75±15 parts per million). The shelf life of premixed tobramycin-containing bone cement is 2 years. In this study all cement was used within 18 months after manufacture and stored at room temperature until use.
Surgical Simplex P bone cement without antibiotics was used as control [Stryker-Howmedica-Osteonics, Rutherford, NJ]

Bacterial strain

Two bacteria were used to contaminate the femur: *S. aureus*, strain Wood-46 (American Type Culture Collection Number 10832) and *S. epidermidis*, strain O-47, [Heilmann, 1996]. The inoculum sizes of *S. aureus* were 10^3, 10^4, 10^5, 10^6 and 10^7 colony-forming units (CFU); those of *S. epidermidis* were 10^8, $10^{8.5}$, 10^9, $10^{9.5}$ and 10^{10} CFU. To prepare the different inocula, the concentration of bacteria in Mueller-Hinton broth had been determined by serial dilution and plating on blood agar. The injection volume of each inoculum was 0.1 ml. Using the E-test, we determined the minimal inhibitory concentration (MIC) of tobramycin to be 0.125 and 0.064 µg/ml, for *S. aureus* Wood-46 and *S. epidermidis* O-47, respectively [Brown, 1991].

Anesthesia

Surgery was performed under strict aseptic conditions and under general inhalation anesthesia. Preoperatively, the rabbits were weighed. The anesthesia was prepared by an intramuscular injection of 4 mg methadone, 4 mg acepromazinemaleate and 0.5 mg atropine. Blood samples on leukocyte counts and erythrocyte sedimentation rate were taken of all rabbits preoperatively. A pressure line was introduced into the auricular artery for measuring blood pressure. Subsequently, the anesthesia was induced by an intravenous injection of 8-12 mg etomidate. An endotracheal tube (#3) was introduced through which the anesthesia was maintained by a 1:1 mixture of nitrous oxide, oxygen and halothane 1%.
Postoperatively, pain relief was provided by 3 mg nalbufine intramuscularly immediately postoperative and subsequently 0.1 mg buprenorfine intramuscularly (if necessary, buprenorfine injection was repeated postoperatively).

Operative technique

The skin of the right leg was clipped and the rabbit was placed with its left side on the table. The operative area was disinfected with povidone-iodine and isolated by sterile drapes. Subsequently, a skin incision (approximately 3

cm) was made over the trochanter tertius of the right femur, parallel to the femoral shaft. The trochanter tertius was exposed by splitting the fascia, retracting the femoral biceps and coccygeofemoral muscles postero-medially and scraping the periost. Using an air-pressured AO minidrill, the cortex was penetrated by a small drill (diameter 1.2 mm). Subsequently this hole was widened and the femoral canal was reamed with 50-mm drills and fraises up to 4 mm in width until a silicon tube (monitor line, outer diameter 3 mm) could be inserted. Cooled PMMA bone cement (4°C) was vacuum-mixed (Simplex cement vacuum-mixer; Stryker-Howmedica-Osteonics, Rutherford, NJ) at 0.9 bar for 60 s (tobramycin-containing bone cement) or 100 s (plain bone cement) on the surgical table. Subsequently, a 6-ml syringe with a 2.0 cm long silicon tube (outer diameter 3.0 mm, inner diameter 1.5 mm) was filled with cement, weighed, and placed in an adapted device on an applicator gun. During these steps, the medullary canal was washed with sterile physiologic saline, suctioned and inoculated with bacteria. Immediately after inoculation, approximately 1.2 ml of the doughy cement was injected gently into the femoral canal, while the syringe was slowly being retracted. The expansion of the cement sealed of the entrance of the medullary canal at this moment, thereby preventing any spill of the bacterial suspension out of the medullary canal. The cement was allowed to polymerize in situ. The syringe was weighed again to determine the amount of cement injected (mean weight of cement in all rabbits was 1.24 ± 0.16 gram). After wound drainage with sterile saline solution, the fascia, subcutis and cutis were closed with Vicryl 3-0.

Follow-up

Postoperatively, localization of the cement was evaluated by routine antero-posterior and lateral radiographs of the right femur (Figure 1), and the rabbits were allowed to recover in a temperature-controlled recovery cage.

The rabbits were monitored by a daily clinical examination, with special attention to wound healing, the presence of a fracture, eating, activity level and body temperature.

Erythrocyte sedimentation rate in the first hour and leukocyte counts were measured preoperatively and weekly postoperatively.

At day 7 or 28, rabbits were killed with an overdose of pentobarbital sodium intravenously.

Post mortem sample acquisition

Excision of the femora was performed under strict aseptic conditions. The left

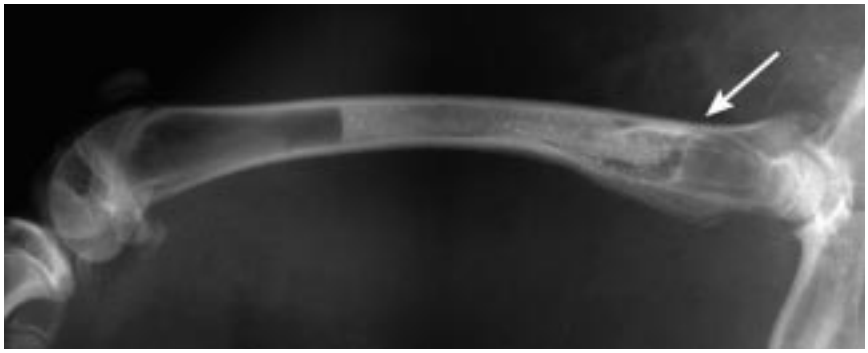


Figure 1. Lateral radiograph of the right femur of a rabbit, which shows the injected cement *in situ*. The arrow indicates the site of injection of the cement.

femur (no bacteria, no cement) was excised to study contamination during the culture procedure. The skin of the hindlimbs was clipped and, with the rabbit placed in prone position on the table, disinfected with a 2% tincture of iodine and isolated by sterile drapes. The left (control) and right femur were excised from all animals and tissue debris was cleaned off. First, a bone fragment was taken from the left femur from a region corresponding to that of the right femur. Then, using a high-speed circular saw, the external surface of the right femur was notched circumferentially at each end of the cement plug and longitudinally on two sides, posterior and anterior. An osteotome was used to free the lateral half of the bone from the medial half. Without damaging the cement plug, the cement was carefully separated from the bone in order to prevent release of tobramycin, which could influence the sampling of bacteria [Powles, 1998]. Only full-thickness cortex adjacent to the cement was used for both bacteriologic culture and histology; bone that was not directly adjacent to the cement was discarded.

Bacteriological culture

All the bone fragments of the lateral half of each femur (in each case approximately 1 g) were ground in a sterile metal mortar. The samples were homogenized in 10 ml of phosphate buffered saline (pH 7.4) using a tissue grinder (Polytron PT 3100 tissue grinder, Kinetica Benelux BV, Best, The Netherlands), 3 minutes at 2500 rpm and subsequently for 5 minutes at 6000 rpm.

A volume of 10 µl of the homogenate (containing 1.0 mg of bone) was then plated on blood agar plates in serial 10-fold dilutions. After an overnight

incubation at 37°C, the samples were counted for viable bacteria. For each femur the number of viable bacteria per gram of bone was calculated (minimum 1000 CFU/g of bone). Infection was defined as a positive culture.

Histology

The medial half of the bone adjacent to the bone cement was used for histological evaluation and fixed in 4% buffered formalin. After decalcification and dehydration the cortex was embedded in paraffin and sectioned on a microtome (Reichert-Jung 2030, Biocut, Leica, Rijswijk, The Netherlands). The sections were mounted on slides and stained with hematoxylin and eosin.

Statistical analysis

The results of the bacteriological cultures were analyzed using the SPSS (Statistical Package for the Social Sciences) version 6.1 for the Macintosh. Differences were considered significant at $p \leq 0.05$. Animals with a comparable history were grouped. To each group one of the five inoculum doses and one of the two follow-up periods were assigned. One half of each group received tobramycin, the other half not, which formed the controls. For each animal a response of the 0/1 type was recorded (negative or positive culture) and the number of cultured bacteria. In the first case, a Mantel-Haenszel type of analysis is called for with a test for trend over the strata (inoculum doses), which was carried out using logistic regression. Differences in response over the strata may be caused by differences between groups. It is, however, unlikely that this would exactly be like a trend, which is more plausibly a dose effect. In the second case a two-way analysis of variance was used to investigate the effect of follow-up and inoculum dose. In particular, a positive relationship of the inoculum dose with the number of cultured bacteria was analyzed by means of polynomial contrasts.

Results

General

In the *S. aureus group,* one rabbit with tobramycin-containing bone cement (7-days follow-up group, inoculum 10^5 CFU) and one control (10^7 CFU, 28-days follow-up group) died just after the cementing procedure. The controls

showed a loss of body weight and elevated erythrocyte sedimentation rate at 7 days. At 28 days these parameters had returned to normal, but the macroscopical aspect of the right femur of these rabbits was suggestive for a local osteomyelitic process (thickened and soft cortex, pus). The *S. epidermidis* controls, as well as all rabbits receiving tobramycin-cement recovered well from surgery and did not develop clinical signs of infection (erythrocyte sedimentation rate, leukocyte count, body temperature and body weight remained normal).

Bacteriological cultures

No positive cultures were found in any of the rabbits receiving tobramycin-cement (Figure 2a and 2b). Twenty-seven out of 29 of the *S. aureus* controls and 16 out of 30 of the *S. epidermidis* controls had positive bacterial cultures. One control rabbit in the 7-days follow-up group that had received 10^7 CFU of *S. aureus* was killed after 5 days, because it was severely ill. The results of the culture of its right femur (^{10}log 5.89) was included as a 7-days result. Table 1 shows an overview of the incidence of infection in the various groups. For both staphylococcal species, the prophylactic effect of tobramycin-containing bone cement was significant ($p < 0.001$). Since all tobramycin treated animals (for both follow-up periods and both bacteria species) did not show positive cultures, differences in trend of the inoculum dose between controls and tobramycin treated animals have not been statistically tested. The number of cultured bacteria in the *S. aureus* controls increased significantly from 7 to 28 days follow-up ($p = 0.044$, $F_{1,19} = 4.64$). Testing for a linear relationship [trend of a positive relationship of inoculum with the extent of infection, see Figure 2(a)]. showed a significant difference of inoculum size ($p = 0.034$, one-sided). For the *S. epidermidis* controls, a significantly lower number of bacteria was cultured after 28 days as compared to 7 days ($p = 0.07$, $F_{1,20} = 9.06$). The effect of the inoculum concentration (linear relationship) was not significant ($p = 0.082$).
All cultures from left femora (not operated on) were negative.

Histology

In the *S. aureus* group, the majority of rabbits in the control group showed a moderate periosteal reaction of the femoral cortex next to the cement after 7 days (Figure 3a). At 28 days follow-up, the *S. aureus* controls showed besides a more severe periosteal reaction also other signs for infection like destruction of the cortex, enlarged Haversian canals and a leukocytes infiltrate (Figure 3b). These changes were also present in the *S. epidermidis* controls,

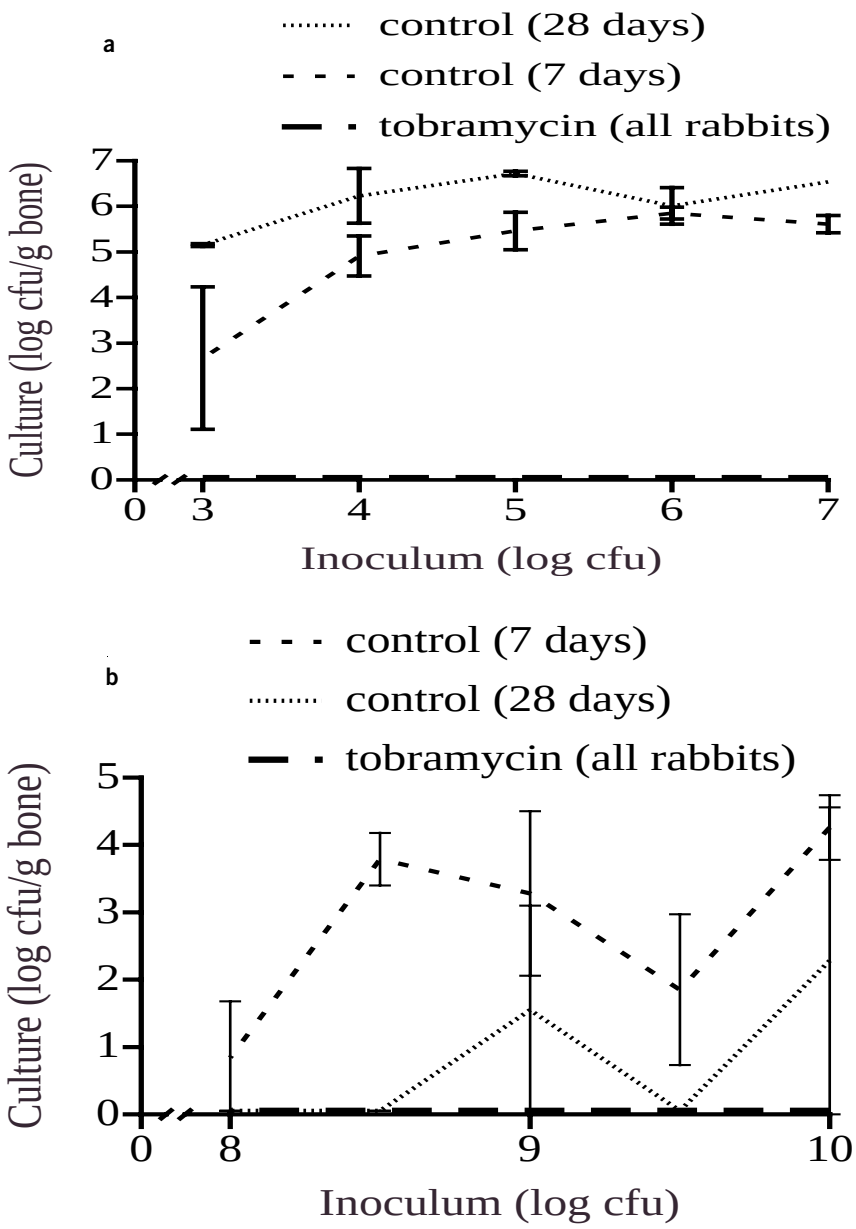


Figure 2. **(a)** Results of cultures (mean ± SEM) of rabbits contaminated with *S. aureus*.
(b) Results of cultures (mean ± SEM) of rabbits contaminated with *S. epidermidis*.

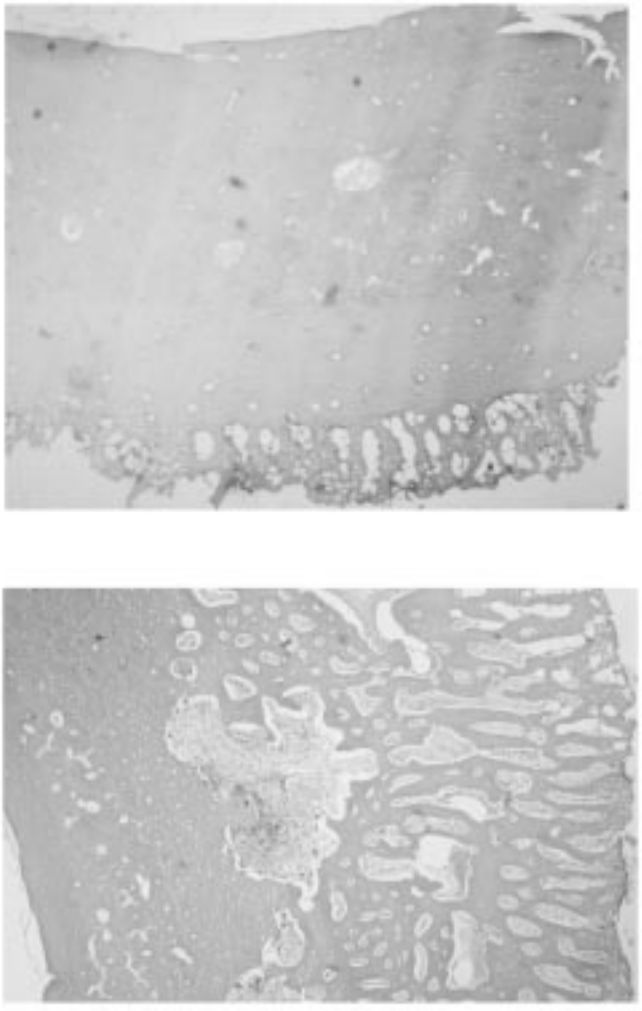


Figure 3. Photomicrographs (hematoxylin and eosin, 50x) of tissue sections of control rabbits contaminated with *S. aureus* showing **(a)** minimal periosteal reaction after 7 days and **(b)** severe periosteal thickening, destruction of cortex, leukocyte infiltrate and granulation tissue after 28 days.

but were less pronounced. We found no histological signs of infection in any of the rabbits that received tobramycin-containing bone cement (Figure 4).

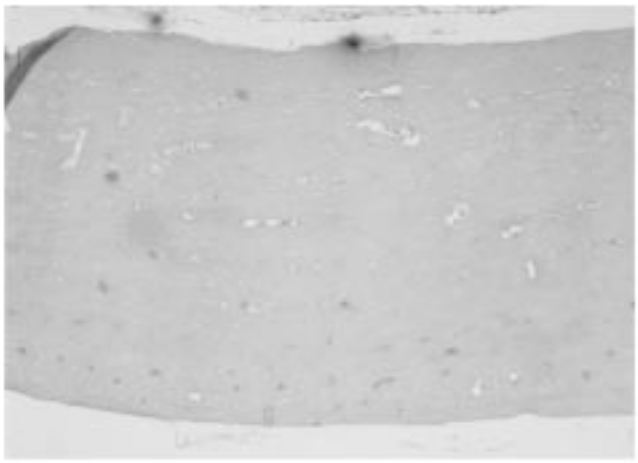


Figure 4. Photomicrograph (hematoxylin and eosin, 50x) of a tissue section from a rabbit with tobramycin-containing bone cement contaminated with *S. aureus* showing no signs of infection after 28 days.

Table 1a. Incidence of infections in the *S. aureus* groups

	7 days		28 days	
Inoculum (^{10}log CFU)	tobramycin	control	tobramycin	control
3	0/4	2/4	0/2	2/2
4	0/4	4/4	0/2	2/2
5	0/3	4/4	0/2	2/2
6	0/4	4/4	0/2	2/2
7	0/4	4/4	0/2	1/1
Total	0/19	18/20 (90%)	0/10	9/9 (100%)

Table 1b. Incidence of infections in the *S. epidermidis* groups

	7 days		28 days	
Inoculum (^{10}log CFU)	tobramycin	control	tobramycin	control
8	0/4	1/4	0/2	0/2
8.5	0/4	4/4	0/2	0/2
9	0/4	3/4	0/2	1/2
9.5	0/4	2/4	0/2	0/2
10	0/4	4/4	0/2	1/2
Total	0/20	14/20 (70%)	0/10	2/10 (20%)

Discussion

In the present study we showed that premixed tobramycin containing-bone cement did prevent infection after inoculation of *S. aureus* and *S. epidermidis*. These findings support our previous study, which showed that the release of tobramycin from this cement, 28 days after insertion, is still more than 40 times the minimal inhibitory concentrations for these staphylococci [Nijhof, 1997]. However, tests of susceptibility based on minimal inhibitory concentration may not be accurate for biomaterial-adherent bacteria and, therefore, *in vivo* studies are required [Naylor, 1990]. Previously, other infection studies in the rabbit, rat, or dog have shown the efficacy of bone cements containing gentamicin or erythromycin/colistin [Elson, 1977b, Petty, 1988, Rodeheaver, 1983, Schurman, 1978]. The efficacy of these antibiotic-bone cement combinations is less clear in the clinical setting: Lynch *et al*. found the efficacy of the gentamicin loaded cement to be statistically proven only in revision operations, not in primary arthroplasties [Lynch, 1987]. Josefsson *et al*. compared the prophylactic efficacy of systemic antibiotics vs. gentamicin bone cement. The difference in favor of the antibiotic bone cement group had lost its statistical difference at 10 years follow-up [Josefsson, 1990, Josefsson, 1993]. Results from the Norwegian arthroplasty show that the additional use of antibiotic bone cement can reduce the infection rate compared to systemic antibiotic only [Espehaug, 1997].

The advantage of antibiotic-containing bone cement is the high local release of antibiotic, which can overcome the decreased susceptibility of bacteria growing on an infected prosthesis. High local release of aminoglycosides like tobramycin increases the rate of bacterial killing, because of their concentration-dependent antibacterial activity [Lacy, 1998]. Eckman *et al*. found effective wound and serum levels of tobramycin in patients with compound fractures after prophylactic treatment with tobramycin-impregnated PMMA beads [Eckman, 1988]. No acute wound infections were seen in these 70 patients. Lyons *et al*. showed that tobramycin-PMMA beads prevented adherence of *S. aureus* in a rabbit model of a contaminated fracture [Lyons, 1992]. The release from hand-mixed tobramycin bone cement has been investigated in primary total hip arthroplasties, but these studies did not mention whether infections were actually prevented [Brien, 1993, Pritchett, 1992]. In another study, infection recurred in one out of ten patients, treated with revision total hip arthroplasty using bone cement hand-mixed with tobramycin (0.5 g tobramycin per unit cement) [Soto-Hall, 1983]. Perioperatively, no systemic antibiotic had been given in this patient, who had intraoperative positive cultures for *Pseudomonas aeruginosa*. Hofmann *et al*. treated 26 infected total knee arthroplasties using hand-mixed tobramycin bone cement (both for the spacer and fixation of the revision prosthesis) together with systemic antibiotics [Hofmann, 1995a]. After a mean follow-up of 30 months, no infection recurred.

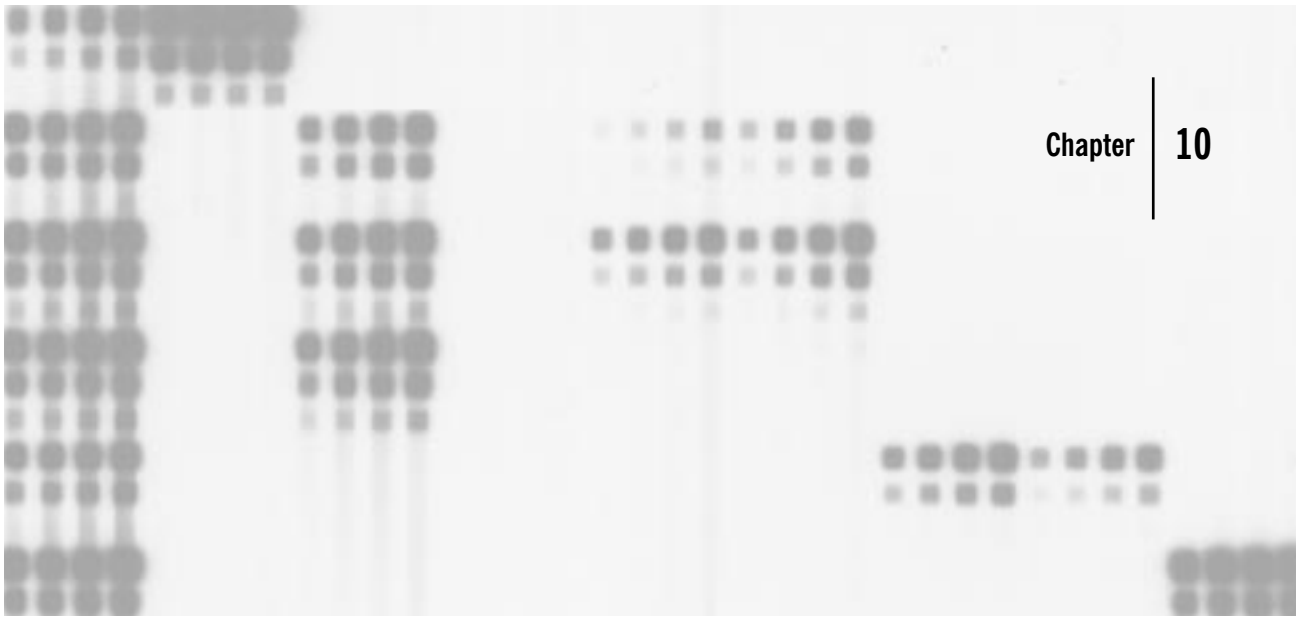
The *S. aureus* controls showed an inoculum-size dependent infection, which increased up to 28 days follow-up. In contrast, the virulence of *S. epidermidis* appeared to be lower, given the higher inoculum dose needed to establish an infection and the lower incidence of infection. *S. aureus* produces a number of extracellular and cell-associated virulence factors, like enterotoxins, collagen- and fibronectin-specific receptors and exopolysaccharides, and probably, therefore, causes more evident signs of infection than *S. epidermidis* [Cunningham, 1996]. *S. epidermidis* is known to adhere preferably to polymers like PMMA [Barth, 1989, Gristina, 1996]. This capability of coagulase-negative staphylococci to colonize and produce a biofilm on a biomaterial is probably an important virulence factor in causing foreign body infections, which infections are more indolent by nature.

The initial loss of two rabbits after cementing probably was caused by pulmonary embolism, due to the increased intramedullary pressure during injection of the cement [Nijhof, 1997]. Thorough lavage of the femoral canal and testing of the width of the reamed femur prevented this complication as much as possible.

It should be noted that, because the method used to culture and count bacteria has a detection limit of around 1000 CFU per gram of bone, very low numbers of bacteria could not be detected. All negative cultures were considered as '0' bacteria present in the sample, whereas theoretically a negative culture can be any culture below 1000 CFU. This might explain the relative large standard errors at some inoculum doses in the control group. Such a minimal detection limit is a relative drawback of all studies that evaluate implant-related infections using a comparable plating and counting technique [Cordero, 1996, Cremieux, 1996, Curtis, 1995, Eerenberg, 1994, Fitzgerald, 1983, Isiklar, 1996, Korkusuz, 1993, Melcher, 1994, Rodeheaver, 1983]. However, many studies do not mention the minimal detectable level of bacteria. Early experiments have shown that in the presence of a foreign body, a number of 100 CFU of a staphylococcal strain is enough to produce an infection in man [Elek, 1957]. Waldvogel *et al*. stated that, because even clean wounds were found to be contaminated, the control of infection is more a quantitative than a qualitative problem [Waldvogel, 1991]. Therefore, the value of antibiotic bone cement is to eliminate the bacteria to such low numbers that host defense mechanisms can clear the remaining infection. It is noteworthy that in present study, the histological appearance of the rabbits with tobramycin-containing bone cement confirmed the negative bacteriological cultures. Especially, the recovery of variant and resistant strains such as small colony variants may be even more difficult due to their altered growth mode [von Eiff, 1997]. It has been reported that both *S. aureus* and coagulase-negative staphylococci can develop resistance against gentamicin after use of gentamicin-containing cement or beads [Hope, 1989, von Eiff, 1997]. Adequate diagnosis of infection is a prerequisite to evaluate new biomaterials and operative techniques optimally, and, of course, to treat patients with an infected implant successfully.

In conclusion, this animal model shows that premixed tobramycin-containing bone cement can prevent implant bed infection in rabbits after contamination with *S. aureus* Wood-46 and *S. epidermidis* O-47.

Prevention of infection with tobramycin-containing bone cement or systemic cefazolin in an animal model



Chapter 10

I Introduction

Adequate infection prophylaxis is mandatory in arthroplasty surgery. Methods to reduce the sources of bacterial contamination in the operating theatre (like ultraclean air, waterproof gowns, gloves, and adhesive plastic drapes) are useful but not perfect [Davis, 1999]. Contamination can still occur and antibiotics may be indicated to prevent infections. In the literature, the optimal mode of administration of prophylactic antibiotics is still subject of discussion. Dutch guidelines state that there is no indication for the use of antibiotic-containing bone cement in primary arthroplasty, if operated under prophylaxis of systemic antibiotics and an ultra-clean air system [Dutch Institute for healthcare improvement (CBO), 1994 #448]. Data from the Swedish hip arthroplasty registry show an increased use of antibiotic-containing bone cement in primary hip arthroplasty from approximately 10% of all primary hip arthroplasties performed in 1978 to 80% in 1996 [Swedish-National-Hip-Arthroplasty-Registry, 1998]. The different modes of administration of antibiotics have been compared for efficacy by only a few experimental and clinical studies. Petty *et al*. showed in a study in dogs that systemic antibiotic treatment as well as local treatment with antibiotic-containing bone cement reduced infections of the implant bed, but only the latter was found to be significantly different from controls [Petty, 1988]. Josefsson *et al*. compared prophylaxis with systemic antibiotics versus gentamicin bone cement in total hip arthroplasty in a prospective randomized clinical trial [Josefsson, 1990, Josefsson, 1993]. At 5-years follow-up, significantly more infections occurred in the group receiving systemic antibiotics. However, at 10-years follow-up of 1688 hips, infection rates in the systemic antibiotics group and in the antibiotic-containing bone cement group were no longer significantly different. In a similar study, McQueen *et al*. found no difference in these two modes of infection prophylaxis in 401 patients at two years' follow-up [McQueen, 1990].

It is obvious from the few available studies that there is still a lack of scientific proof regarding the efficacy of systemic (intravenous) versus local (bone cement) administration of antibiotics to prevent implant bed infection. In the present study, we investigated the efficacy of prophylaxis either with intravenously administered cefazolin or by use of tobramycin-containing bone cement in an experimentally infected implant bed. We chose these two types of antibiotic with different routes of administration (systemic and local) because of their clinical relevance. Cefazolin is a first-generation

cephalosporin and is used widely by orthopedic surgeons for treatment of staphylococcal infections. It has a longer half-life and provides for higher serum concentrations than the other first-generation cephalosporins. Tobramycin-containing bone cement has previously been shown to be efficacious in prevention of infections both *in vitro* and *in vivo* in rabbits [Nijhof, 1999, Scott, 1999]. For this type of cement, no data are available on its efficacy as a prophylactic treatment in comparison with systemic antibiotics. Therefore, the aim of the present study was to investigate the efficacy of tobramycin-containing bone cement and systemic cefazolin in preventing infection in a rabbit model.

Materials and Methods

Design

In a total of 18 rabbits, the femoral cavity was inoculated with 10^6 colony-forming units (CFUs) of *Staphylococcus aureus*. Six rabbits (group A - systemic antibiotic) received an intravenous injection of cefazolin before inoculation. Subsequently, plain Simplex-P bone cement (Stryker-Howmedica-Osteonics, Rutherford, NJ) was injected into the femoral cavity. Six rabbits (group B - tobramycin cement) received only Simplex-P tobramycin-containing bone cement (Stryker-Howmedica-Osteonics, Rutherford, NJ) after local inoculation of the femoral canal. Six rabbits (group C - control) did not receive any antibiotic treatment, and plain Simplex-P bone cement was injected in the femur after local inoculation. Seven days after surgery, the animals were killed and the femoral cortex adjacent to the cement was cultured. The efficacy of the three treatments was assessed based on the number of CFUs of the bacteria and on the detection of *S. aureus* DNA by a polymerase chain reaction (PCR) hybridization assay. The guidelines according to the Dutch act on animal experiments (1985) were observed.

Bacterial strain

S. aureus, strain Wood-46 (ATCC 10832) was used to inoculate the rabbit's femur. After culture in Mueller-Hinton broth, a stock of aliquots was frozen. The concentration of bacteria was determined by serial dilution and plating on blood agar. Preoperatively, samples containing 10^7 CFU/ml were prepared. A volume of 0.1 ml (10^6 CFU) was injected into the rabbit femoral cavity.

Previous studies have shown this dosage to result in a 100% infection rate of cement bodies [Nijhof, 2000a].

Surgery

Healthy adult female New Zealand white rabbits (Ico: NZW) weighing 3000-3500 gram were obtained one week before surgery to acclimatize them to the housing in the Central Animal Laboratory. They were fed daily with 80-100 gram antibiotics-free rabbit diet and water *ad libitum*.

Preoperatively the rabbits were weighed. The systemic antibiotic (cefazolin, 30 mg/kg) was injected 30 minutes before surgery, into the left auricular vein of the rabbits in group A. The anesthesia was prepared by an intramuscular injection of 4 mg methadone, 4 mg acepromazinemaleate and 0.5 mg atropine. A preoperative blood sample was taken from the left auricular vein, 5 minutes before surgery. A pressure line was introduced into the auricular artery for measuring blood pressure. Subsequently the anesthesia was induced by an intravenous injection of etomidate (8-12 mg). An endotracheal tube was introduced through which the anesthesia is maintained by a 1:1 mixture of nitrous oxide, oxygen and halothane 1%. The skin of the outer right thigh was clipped and the rabbit was placed with its left side on the table.

The operative area was disinfected with povidone-iodine and isolated by sterile drapes. Subsequently, a skin incision (approximately 3 cm) was made parallel to the femur shaft, over the trochanter tertius of the right femur. The trochanter tertius was exposed by splitting the fascia, retracting the femoral biceps and coccygeofemoral muscles posterior-medial and scraping the periost. The cortex was penetrated with a small drill (diameter 1.2 mm), using an air-pressured AO minidrill. Subsequently, the femoral canal is reamed up to 4.0 mm in width. The content of the medullary canal was suctioned. Cooled (4°C) sterile bone cement was vacuum-mixed on the surgical table. The rabbits received either plain Simplex-P bone cement (groups A and C) or tobramycin-containing Simplex-P bone cement (group B). After injection of 0.1 ml of the bacteria suspension into the femoral canal, approximately 1.2 ml of cement was inserted. The exact amount of inserted cement was determined by weighing the syringe containing the cement. The fascia, subcutis, and cutis were closed with Vicryl 3-0 after polymerization of the cement and wound drainage with saline. Pain relief was provided by intramuscular injection of 3.0 mg nalbufine immediately postoperatively and subsequently 0.1 mg buprenorfine. Buprenorfine injection was repeated when necessary.

Follow-up

Postoperatively, routine AP and lateral X-rays were made of the right femur. The rabbits recovered in a temperature controlled recovery cage. The rabbits were monitored by a daily clinical examination, with special attention for wound healing, the presence of a fracture, eating, activity level, and body temperature. The erythrocyte sedimentation rate (ESR) and white blood cell counts (WBC) were measured before surgery and 1 and 7 days after. After 7 days, the rabbits were killed with an intravenously administered overdose of pentobarbital N2.

Autopsy and sample acquisition

After the animals were killed, the skin of the left and right thighs was clipped, disinfected with povidone-iodine, and isolated with sterile drapes. The right and left (control) femora from all animals were excised and cleaned from tissue debris. Using a high-speed dental drill with a circular diamond saw, the external surface of the right femur was notched circumferentially at each end of the shaft and longitudinally on two sides. A mallet and an osteotome were used to break off each metaphysis and then to free the lateral half of cortex adjacent to the cement. Care was taken not to damage the cement. The bone samples from the left femur were taken from the site corresponding to the right femur operation site.

Bacteriological examination

The lateral half of cortex adjacent to the cement plug (in the right femoral canal) and bone from the corresponding site of the left femur were submitted for quantification of bacteria. For this purpose, the bone samples of approximately 1 g were cut into small pieces and homogenized in 10 ml phosphate-buffered saline (pH 7.4) using a Polytron tissue grinder. Subsequently, the number of bacteria (CFU) per gram of bone was determined by dilution and plating on blood-agar plates.

Histology

The medial half of the bone was used for histological evaluation and fixed in 4% buffered formalin. After decalcification and dehydration the cortex was embedded in paraffin and sectioned on a microtome (Reichert-Jung 2030; Biocut, Leica, Rijswijk, The Netherlands). The sections were mounted on slides and stained with hematoxylin and eosin.

PCR hybridization assay

A part of the lateral half of the right femoral cortex (mean weight 0.24 g) adjacent to the cement plug was collected for molecular biological analysis for the presence of bacterial DNA. Samples were incubated for 18 hours at 60°C in digestion buffer [500 m*M* Tris (pH 9), 20 m*M* ethylenediamine tetraacetic acid (EDTA), 10 m*M* NaCl, 1% sodium dodecyl sulfate(SDS), 0.5 mg/ml proteinase K]. to release total DNA. DNA was isolated using a PCR purification kit (Qiagen, Hilden, Germany). DNA was amplified by the technique described by Wilbrink *et al* [Wilbrink, 1998]. Broad-range biotin-labeled primers, targeting conserved regions of the 16S-rRNA gene, were used to set up an eubacteria-specific PCR. An internal spike was added to screen for possible inhibition of PCR and to reduce the amplification of contaminating DNA. The presence of *S. aureus* DNA was determined by reverse line blot hybridization (RLB). We used the RLB technique as described by Kaufhold *et al* [Kaufhold, 1994]. For this purpose, we used a genus-specific staphylococcal oligonucleotide probe (5'-AACCTACCTATAAGACTGG-3') and a species-specific *S. aureus* oligonucleotide probe (5'-TCAAAAGTGAAAGACG-GTC-3') which were covalently linked to a membrane (Biodyne C; Pall Biosupport, Portsmouth, UK). PCR products were hybridized to the oligonucleotide probes on the membrane for 1 hour at 42°C, using a miniblotter system (MN45, Immunetics, Cambridge, MA). Subsequently, nonspecific DNA was washed of the membrane at 55°C and the membrane was incubated at 42°C with Streptavidin-peroxidase (Boehringer Mannheim Biochemica, Mannheim, Germany). Finally, the presence of *S. aureus* DNA was visualized on a film (Hyperfilm ECL) using an enhanced chemoluminescent detection system (ECL; Amersham international, Little Chalfont, England).

Results

All rabbits recovered well from surgery. The inserted cement [mean ± standard deviation (SD)]. weighed 1.46 ± 0.09 gram in group A (tobramycin cement), 1.28 ± 0.18 gram in group B (systemic antibiotic), and 1.29 ± 0.17 gram in group C (control).

The loss of body weight (mean ± SD) of the rabbits at 7 days was 106 ± 101 gram (3.7% as a percentage of their initial body weight) in group A, 65 ± 52 gram (2.1%) in group B and 246 ± 109 gram (8.5%) in group C.

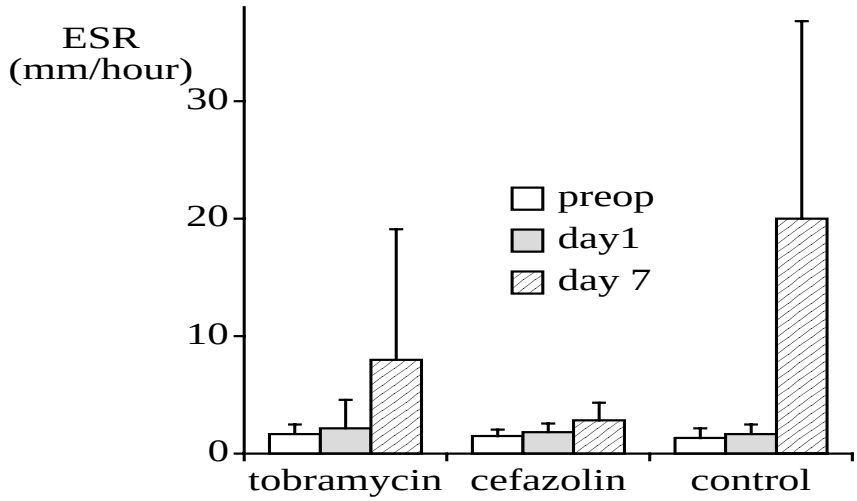


Figure 1. Erythrocyte sedimentation rates (mean) in the antibiotic treated groups and the untreated control group, at three different time points (preoperatively and 1 and 7 days' follow-up). Error bars represent standard deviation.

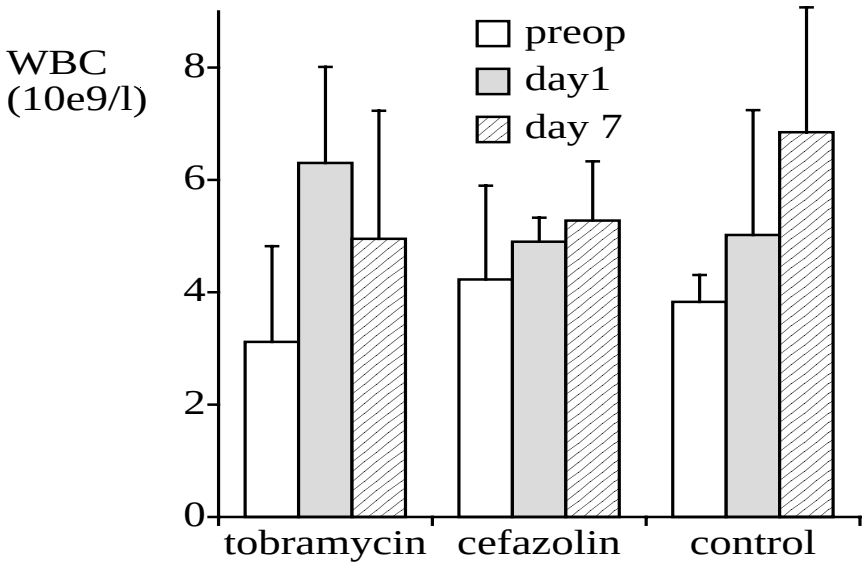


Figure 2. White blood cell counts (mean) in the antibiotic-treated groups and the untreated control group, at three different time points (preoperatively and 1 and 7 days' follow-up). Error bars represent standard deviation.

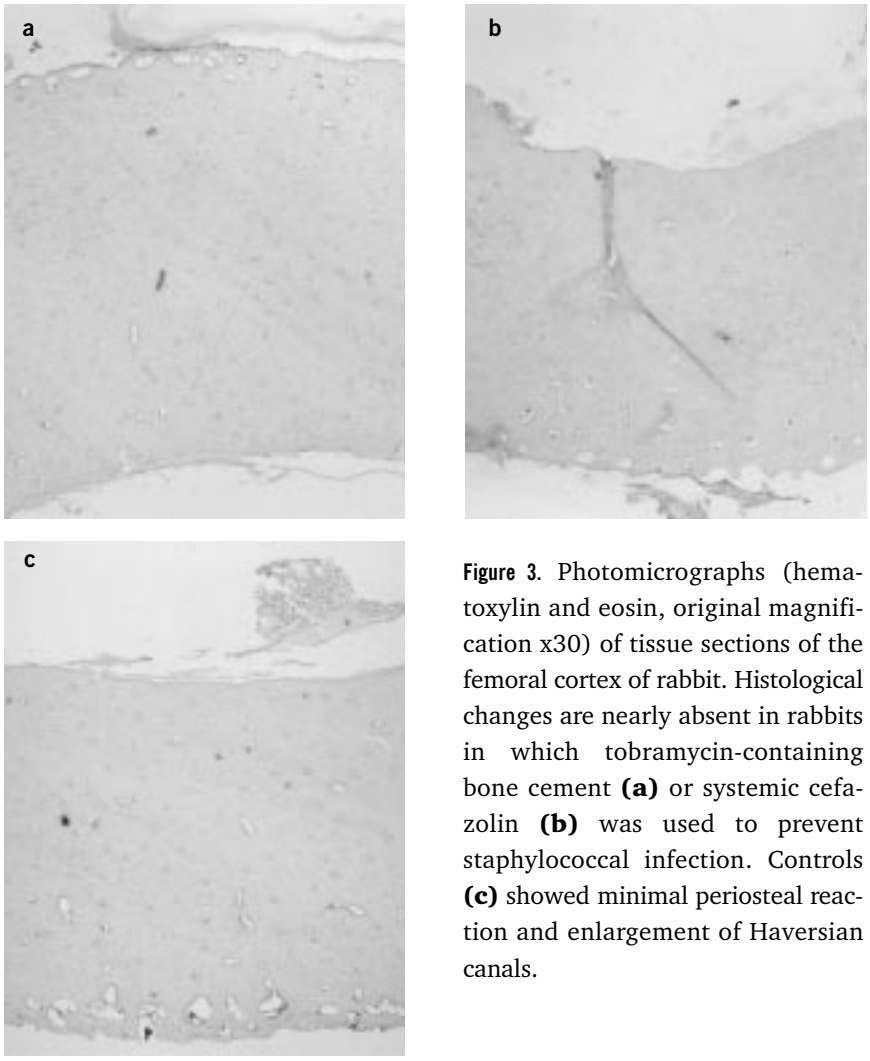


Figure 3. Photomicrographs (hematoxylin and eosin, original magnification x30) of tissue sections of the femoral cortex of rabbit. Histological changes are nearly absent in rabbits in which tobramycin-containing bone cement **(a)** or systemic cefazolin **(b)** was used to prevent staphylococcal infection. Controls **(c)** showed minimal periosteal reaction and enlargement of Haversian canals.

The ESR was elevated at 7 days' follow-up, especially in the control group (Figure 1). The elevation of ESR was less in both antibiotic groups. Leukocyte counts were not different between the three groups (Figure 2).

Cultures from the rabbits which received antibiotic prophylaxis (either cefazolin systemically or tobramycin-containing bone cement) were all negative. In contrast, all six rabbits in the control group (plain bone cement, no antibiotics) had positive cultures (Table 1). In all rabbits, cultures from the left femur (not operated on) were negative.

Histology of sections of the right femur showed no marked differences

Table 1. Results of culture of right femoral cortex

Group of rabbits	Incidence of infection	Culture (^{10}log CFU/g, mean ± SD)
Tobramycin cement	0/6	0
Cefazolin systemically	0/6	0
Controls	6/6	5.39 ±0.79

between the three groups with regard to signs of infection. In sections of the control group only minimal elevation of the periost and enlargement of Haversian canals was seen, with no destruction of the cortex or increase in leukocytes (Figure 3a-c).

Using the PCR-hybridization assay, the presence of *S. aureus* DNA was identified in the right femur of all the rabbits in the control group (Figure 4). In contrast, the femur samples from groups that received antibiotic prophylaxis, either cefazolin systemically or tobramycin-containing bone cement, *S. aureus* DNA was not detectable.

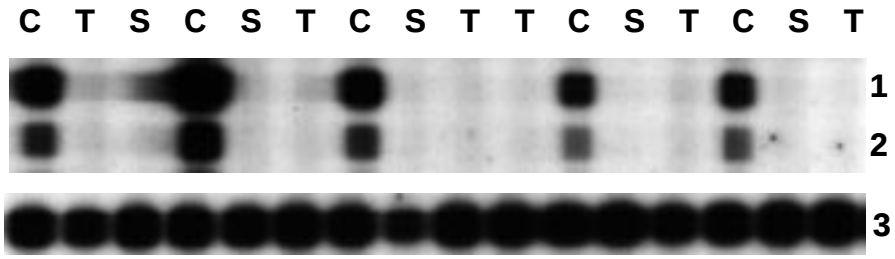


Figure 4. Details of the film with the results of the reverse line blot hybridization assay. PCR products of the right tibiae of the rabbits in the three different treatment groups are oriented in vertical lanes (T = tobramycin group, C = control group, S = systemic cefazolin group). The oligonucleotides are oriented in horizontal lanes (1 = staphylococci probe, 2 = *S. aureus* probe, 3 = internal spike probe). An internal spike was added to all samples to exclude possible inhibition: The lower lane shows no inhibition in samples that were negative for staphylococci or *S. aureus*.

Discussion

In the present study, we demonstrated that both tobramycin-containing bone cement and systemically administered antibiotic can prevent infection of the rabbit's femur after inoculation with *S. aureus*. Nielsen *et al*. obtained similar results comparing the use of gentamicin-impregnated bone cement with systemic administration of dicloxacillin in a rabbit model [Nielsen, 1996]. In a canine model, Petty *et al*. compared the prophylactic efficacy of adding gentamicin to bone cement with other antibiotic treatment modalities, including the use of intravenous cefazolin [Petty, 1988]. The prophylactic effect of the use of gentamicin-impregnated bone cement was absolute; that of the use of intravenous cefazolin was not. In contrast to their results, we did not see any infection in rabbits, which received cefazolin systemically to prevent local infection. Because details of their infection model regarding the inoculum size, volume of inserted cement and type of bacterial strain used were not provided, it is difficult to explain the different results obtained in their model. The prophylactic effect of systemic antibiotics may depend on the animal species used, because in another rabbit model, a single preoperative dose of cefazolin prevented *S. aureus* infection during spinal instrumentation [Guiboux, 1998]. Another factor that influences the outcome of an infection model is the timing and mode of administration of the inoculum. Blomgren and Elson *et al*. studied the effect of gentamicin-impregnated bone cement on haematogenous infection in experimental models [Blomgren, 1981, Elson, 1977b]. When the inoculum was administered intravenously, 6 weeks after the initial operation, both authors found no significant difference in the incidence of infection whether or not gentamicin was used in the bone cement. The antibacterial effect of gentamicin could was shown only when the haematogenous inoculation occurred immediately after wound closure [Elson, 1977b]. Even when a knee arthroplasty in rabbits is inoculated via an intra-articular injection seven days after implantation, an infection is difficult to initiate [Schurman, 1978]. As a consequence of this, Schurman *et al*. could not show a persistent prophylactic effect up to 1 week of bone cement containing gentamicin.

The infection rate after joint replacement surgery can be influenced by many prophylactic methods. Guidelines for prevention of infection differ between hospitals, mainly due to the fact that it is hard to prove the effect of an individual factor contributing to the reduction of infection rate after surgery.

Clinically, randomized prospective studies evaluating such factors need to include many patients, and as a consequence, it is difficult to control the contribution of one factor when studying the other [Fitzgerald, 1992, Hill, 1981, Lidwell, 1982, Salvati, 1982a]. In addition, the generalizability of the results of randomized trials is often low owing to restrictive patient selection in these trials or to the fact that results obtained in centers of excellence are often not representative of results in the community [Herberts, 1997].

In the literature, the debate as to whether to use antibiotic-containing bone cement or systemic antibiotics for prophylaxis of arthroplasty infection is not yet concluded [Josefsson, 1993, McQueen, 1990, Petty, 1988]. The difficulties in correctly diagnosing deep infection have prompted authors to reclassify their results at longer follow-up periods [Josefsson, 1993]. The long-term follow-up studies of Josefsson *et al*. illustrated nicely that for some patients it took years for the infection to manifest itself, whereas others' signs and symptoms of infection had to be reinterpreted [Josefsson, 1990, Josefsson, 1993, Josefsson, 1981]. It might well be that the combination of the two strategies, both antibiotic-containing bone cement and systemic antibiotics, is the optimal choice. Espehaug *et al*. evaluated infection incidence after 10,905 primary cemented hip replacements in Norway, with a follow-up of 8 years [Espehaug, 1997]. The effect of prophylaxis of antibiotics administered systemically, in bone cement, or both was studied. The best results were obtained with the combination therapy. Nowadays, economic arguments could also influence the choice between different strategies in medicine. Persson *et al*. calculated that although the use of antibiotic-containing bone cement as a prophylactic option next to systemic antibiotics can reduce the risk of costly revisions, this might not always be the most cost-effective strategy [Persson, 1999].

In the present study, the minimal detectable level for *S. aureus* was 1000 CFUs per gram of bone. For reasons of reproducibility, we did not choose to concentrate the bacteria in smaller sample volumes after milling of the bone. However, in addition to our dilution and plating method, we also used a PCR-hybridization assay. PCR-based methods for detection of bacterial DNA can improve sensitivity of diagnosis of orthopedic implant infections [Mariani, 1996]. Using a PCR-hybridization assay, we confirmed both the presence of *S. aureus* DNA in the untreated controls, as well as its absence in the two antibiotic-treated groups. These findings convincingly demonstrate the efficacy of both types of antibiotic prophylaxis.

It can be disputed that, although our study provided enough power to reveal differences between the cefazolin or tobramycin and the control group

respectively, the power of actual comparison between the cefazolin and tobramycin groups was low, and therefore we could not arrive at a conclusion regarding differences between the two antibiotic groups. This power is low, because both treatments were efficacious as none of the animals in each group had positive cultures. Thus, such a comparison of two apparently efficacious treatments would require a large number of animals (>100). The question of clinical relevance of data would then arise. In addition, the subsequent PCR-hybridization assay supported our findings that both systemic antibiotic and local tobramycin bone cement are effective for infection prevention.

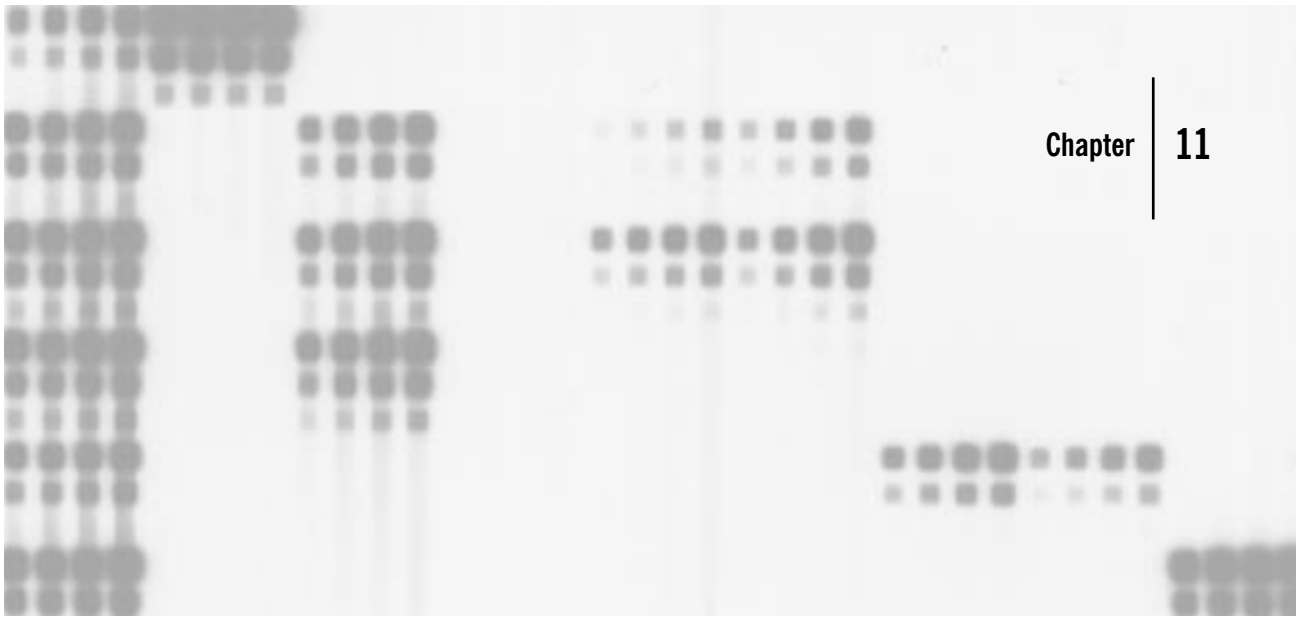
We used *S. aureus* (strain Wood 46), susceptible to both tobramycin and cefazolin, as the infectious agent. Recently, Scott *et al*. showed in an *in vitro* study that some bacterial strains, resistant to the usual systemic concentrations, displayed some degree of susceptibility to tobramycin-containing bone cement [Scott, 1999]. This phenomenon has been addressed by other authors who compared systemic administration of cefazolin with topical cefazolin administration using microspheres [Fallon, 1999]. Similarly, The high local release of antibiotic from antibiotic-containing bone cement might be an advantage compared to systemic antibiotics in preventing infections with resistant strains of bacteria.

This animal model was used to study the development of an infection in the rabbit after initial contamination with a pathogen. This aspect differs from the situation of treating an already existing infection. Thus, this model does not allow for the evaluation of the efficacy of different treatment options for an infected prosthesis, like treatment with a one- or two-stage revision, or by retaining the prosthesis and systemic antibiotics [Buchholz, 1981, Elson, 1998, Isiklar, 1996, Ivarsson, 1994, Lieberman, 1994, Masri, 1998c, McDonald, 1989, Raut, 1995, Ure, 1998]. Study of the efficacy of tobramycin-containing bone cement as a treatment modality is the goal of a future animal study.

In conclusion, we have demonstrated the efficacy of tobramycin-containing bone cement and systemically administered cefazolin in preventing infection in the rabbit, by both culture and detection of bacterial DNA. Possibly, both antibiotic regimes applied together will provide optimal prophylaxis of orthopedic prosthesis infection.

Tobramycin-containing bone cement and systemic cefazolin in a one-stage revision

Treatment of infection in a rabbit model



Chapter 11

I Introduction

Infections of total joint prostheses are frequently associated with the presence of necrotic bone, devascularization and bacteria with specific growth characteristics. Especially the latter, that is the ability of pathogens to adapt to the presence of a foreign body and sustain capability to withstand antibiotics and host defenses, complicates treatment. Therefore, rigorous treatment modalities are necessary to eliminate the infection, but the best choice for treatment of an infected total joint prosthesis still remains to be clarified. Consensus exists among most orthopaedic surgeons to remove the infected prosthesis if possible, because the infection is difficult to treat in presence of foreign material covered with bacteria [Brandt, 1997]. Usually antibiotic-loaded bone cement is used for fixation of a new prosthesis to provide a high local tissue concentration of antibiotics. Such a revision operation can be performed either as a one-stage procedure or as a two-stage procedure. In the one-stage revision the infected implant is removed and, after debridement and lavage of the implant bed, during the same session replaced by a new prosthesis. In a two-stage revision, the insertion of the new implant is postponed until after removal of the infected implant in a time period whereby the infection is treated with systemic antibiotics and/or local antibiotic-loaded beads. The new prosthesis is inserted not until the infection parameters have regained normal levels. The use of antibiotic-loaded bone cement for fixation of the revision prosthesis is preferred given the higher incidence of infection after revision in comparison with primary joint prostheses. Where most surgeons choose the two-stage procedure for exchange of an infected prosthesis, also large series have been reported using only the one-stage procedure, especially in Europe [Buchholz, 1981, Raut, 1995]. So far, reviews of literature on infected arthroplasties reported success rates of 82-83% of one-stage revisions, and 91-93% of two-stage revisions [Garvin, 1995, Pagnano, 1997].
In a previous study, it has been shown that Simplex P tobramycin-containing bone cement can prevent *Staphylococcus aureus* and *Staphylococcus epidermidis* infections in the rabbit's femur [Nijhof, 2000a]. In that model we evaluated the development of an infection after inoculation of the medullary canal with bacteria, immediately followed by insertion of the antibiotic-containing bone cement. However, in order to treat a pre-existent implant infection, one that has developed after introduction of bacteria at an earlier time point, the antibiotic-containing bone cement should also be effective against pathogens that may have become phenotypically adapted to their new habitat. In a one-stage

revision procedure, when no previous surgical attempts have been undertaken to treat such an infection, this may be even more demanding. The purpose of the present study was to compare the efficacy of tobramycin-containing bone cement with that of systemic cefazolin for treatment of infection in a one-stage revision model. In addition to conventional culture techniques a PCR hybridization assay was used in the detection of bacteria as bacterial DNA.

Materials and methods

Design

To establish an infection, a pre-formed non antibiotic-containing cement plug was introduced in the right tibial medullary canal of 30 rabbits, after local inoculation with *S. aureus*. Four weeks after insertion of the implant, the rabbits were divided into three groups (10 rabbits each), and a one-stage revision of the implant was performed in all rabbits. After removal of the infected implant and lavage of the implant bed (without formal debridement), bone cement was injected into the medullary canal. Group 1 received tobramycin-containing bone cement, Group 2 received plain bone cement (no antibiotics) as control, and Group 3 received plain bone cement and additional systemic antibiotics. Fourteen days after the revision procedures the tibiae were excised and the cortex adjacent to the cement was cultured. The efficacy of the different treatments was compared based upon the number colony-forming units of bacteria following culture.

Bacterial strain

Staphylococcus aureus, strain Wood-46 (ATCC 10832) was used. After culture in Mueller-Hinton broth, a stock of aliquots was frozen. The concentration of bacteria (colony-forming units per milliliter, CFU/ml) was determined by serial dilution and plating on blood agar. In a volume of 0.1 ml, a dose of either 10^5 CFU or 10^6 CFU was injected in the medullary canal of the rabbit's tibia. The first 4 rabbits in each group received an inoculum dose of 10^6 CFU. This dose was the same as was used in a previous study on the prevention of implant bed infection in the tibia of rabbits [Nijhof, 2000b]. In an attempt to reduce loss of rabbits due to sepsis the dose was changed to 10^5 CFU in the subsequent rabbits. The latter inoculum dose has proved to establish an infection in another animal model of tibial implant infection [Vogely, 2000b].

Animals

Healthy adult female New Zealand white rabbits (Ico:NZW, Broekman Instituut BV, Someren, The Netherlands) weighing 3000-3500 gram were obtained one week prior to surgery to acclimatize to the housing in the Central Animal Laboratory. The animals were caged in individual cages, fed with 80-100 gram antibiotics free Hope Farms rabbit diet LKK-20 and water *ad libitum*. Postoperatively, the animals were kept in the barrier housing facility of the Central Animal Laboratory until they were killed.

Surgery

The anesthesia protocol was the same for both operations (induction of infection and revision of implant). Surgery was performed under strict aseptic conditions and under general inhalation anesthesia. Preoperatively the rabbits were weighed. The anesthesia was prepared by an intramuscular injection of 4 mg methadone, 4 mg acepromazinemaleate and 0.5 mg atropine. A pressure line was introduced into the auricular artery for measuring blood pressure. Subsequently the anesthesia was induced by an intravenous injection of etomidate (8-12 mg). An endotracheal tube (#3) was introduced through which the anesthesia is maintained by a 1:1 mixture of nitrous oxide, oxygen and halothane 1%. The skin of the right leg was clipped and the rabbit was placed with its left side on the table. The operative area was disinfected with povidone-iodine and isolated by sterile drapes. Postoperatively, pain relief was provided by 3 mg nalbufine i.m. immediately postoperative and subsequently 0.3 mg buprenorfine i.m. If necessary, buprenorfine injection was repeated postoperatively.

Infection of primary implant

At the first operation (establishment of infection), the right knee joint was opened via a parapatellar incision. Anterior to the insertion of the anterior cruciate ligament on the tibia, the medullary canal was opened. Using an air-pressured AO minidrill the cortex was penetrated by a small drill (diameter 1.2 mm) and the medullary canal was reamed with drills and fraises up to a length of at least 25 mm and 3.9 mm in width. The content of the medullary canal was suctioned and flushed with saline. Prior to insertion of the implant, the bacterial suspension was introduced in the tibial canal. Subsequently, the implant (pre-formed cement on a central metal wire, 25 mm in length, 3.9 mm in diameter, Figure 1) was press-fit inserted in the medullary canal. The joint capsule and skin were closed in layers with Vicryl 3-0.

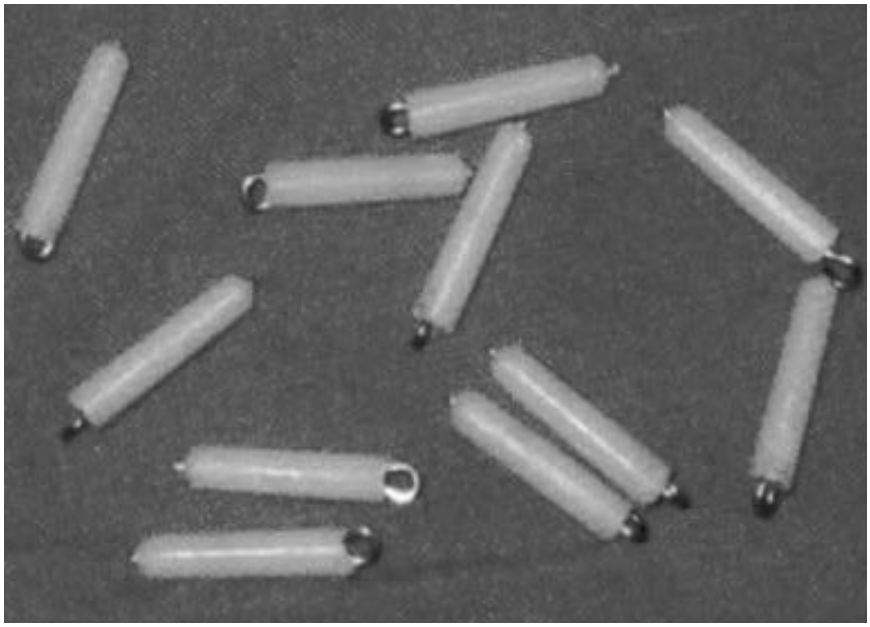


Figure 1. Example of implant used to create an infection.

Revision of implant

The implant was exchanged 28 days after the first operation. Through the parapatellar scar the knee joint was opened. The present implant was removed from the right tibial canal, inoculated on blood agar plates and incubated for 24 hours at 37°C. In addition, medullary tissue samples were obtained for culture. Subsequently, the canal was debrided and washed with sterile antibiotic free physiologic saline. In Group 1, tobramycin-containing bone cement (Simplex P bone cement, premixed with 1.0 g of tobramycin as a sulphate in 40 g powder, Stryker-Howmedica-Osteonics, Rutherford, NJ) was inserted in the right tibial medullary canal. In the same manner, plain Simplex-P bone cement was used in Groups 2 and 3. The animals in Group 3 received also systemic antibiotics (cefazolin, 30 mg/kg, injected subcutaneously every 8 hours for 14 days total, from day 28 through day 42). The rabbits in Group 2 did not receive any form of antibiotic treatment. The bone cement (precooled at 4°C) was vacuum-mixed for 60 seconds (tobramycin-containing bone cement) or 100 seconds (plain bone cement) on the surgical table. Approximately 1.2 ml cement was injected gently into the medullary canal, while the syringe was slowly being retracted. The exact amount of

injected cement was determined by weighing the syringe containing the cement. After polymerization of the cement and wound drainage with saline, the joint capsule and skin were closed in layers with Vicryl 3-0.

Follow-up

The follow-up period after revision surgery was 14 days (42 days after the first operation). Routine AP and lateral X-rays of the right femur were obtained after the first operation and before and after revision surgery on day 28. Body weight and body temperature were recorded on a regular basis. Blood samples from the auricular vein on erythrocyte sedimentation rate (ESR) and white blood cell counts (WBC) were taken prior to the first operation and at day 1, 7, 14, 21, 28 (prior to revision), 35 and 42 postoperatively. The animals were killed with an overdose pentobarbital sodium intravenously.

Autopsy and sample acquisition

After the animals were killed, the skin of the both legs was clipped, disinfected with povidone-iodine and isolated by sterile drapes. The right and left (not operated on) tibia were excised and cleaned from soft tissue debris. First, bone samples were taken from the left tibia from a region corresponding to the right tibia samples. Secondly, the external surface of the right tibia was notched circumferentially at each end of the shaft and longitudinally on two sides, posterior and anterior. An osteotome was used to break off each metaphysis and then to free the medial half of the bone from the lateral half. Care was taken not to damage the cement.

Bacteriological culture

Both the medial bone half of the right tibia adjacent to the cement plug and bone from the corresponding region of the left tibia were submitted for quantification of bacteria. The bone samples were homogenized in a sterile phosphate buffered saline solution (pH 7.4) using a Polytron tissue grinder (Kinetica, Best, The Netherlands) and the number of bacteria per gram of bone was determined by dilution and plating techniques.

PCR hybridization assay

A part of the medial half of the right tibial cortex adjacent to the cement plug was collected for molecular biological analysis for the presence of bacterial DNA. These samples were incubated for 18 hours at 60°C in 1.5 ml digestion buffer (500 mM Tris (pH 9), 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) to release total DNA. A volume of 200 µl of the extracted DNA

was used for DNA isolation the QIAamp Tissue Kit (Qiagen, Hilden, Germany). The last step in the isolation of the DNA included the elution of DNA in an end-volume of 400 µl. Subsequently, 2.5 µl of the dissolved DNA was amplified by the technique described by Wilbrink *et al.* [Wilbrink, 1998]. Broad range biotin-labeled primers, targeting conserved regions of the gene for the 16S subunit of ribosomal RNA (16S rRNA), were used to set up an eubacteria-specific polymerase chain reaction (PCR). An internal spike was added to screen for possible inhibition of PCR and to reduce the amplification of contaminating DNA. The presence of *S. aureus* DNA was determined by reverse line blot hybridization. We used the reverse line blot hybridization technique as described by Kaufhold *et al.* [Kaufhold, 1994]. For this purpose, we used a genus-specific staphylococcal oligonucleotide probe (5'-AACCTAC-CTATAAGACTGG-3') and a species-specific *S. aureus* oligonucleotide probe (5'-TCAAAAGTGAAAGACGGTC-3') which were covalently linked to a membrane (Biodyne C, Pall Biosupport, Portsmouth, UK). Ten microliter of PCR products were hybridized to the oligonucleotide probes on the membrane for 1 hr at 42 °C, using a miniblotter system (MN45, Immunetics, Cambridge, MA). Subsequently, nonspecific DNA was washed of the membrane at 55 °C and the membrane was incubated at 42 °C with Streptavidin-peroxidase (Boehringer Mannheim Biochemica, Mannheim, Germany). Finally, the presence of *S. aureus* DNA could be visualized on a film (Hyperfilm ECL) by using an enhanced chemoluminescent detection system (ECL, Amersham international, Little Chalfont, England).

Statistics

The probability of a positive culture was compared between the three groups of rabbits using the Fisher's exact test. Furthermore, to account for inoculum dose, also a more sophisticated analysis by using a stratified two by two chi-squared test was performed. One-sided tests were performed for comparison of each antibiotic group (tobramycin-bone cement and systemic cefazolin) with the control group. Two-sided tests were performed for comparison of the two antibiotic groups. A P-value of less than 0.05 was considered significant. Exact P-values have been computed using the statistical program StatXact 4.

I Results

General

Three rabbits were lost before the time of revision. Two of these rabbits died in the first week after the initial operation, the third showed signs of severe sepsis and was killed in the third postoperative week. In all three rabbits the culture revealed an overwhelming *S. aureus* infection at the side of the implant. All other rabbits had a good recovery from both operations. The

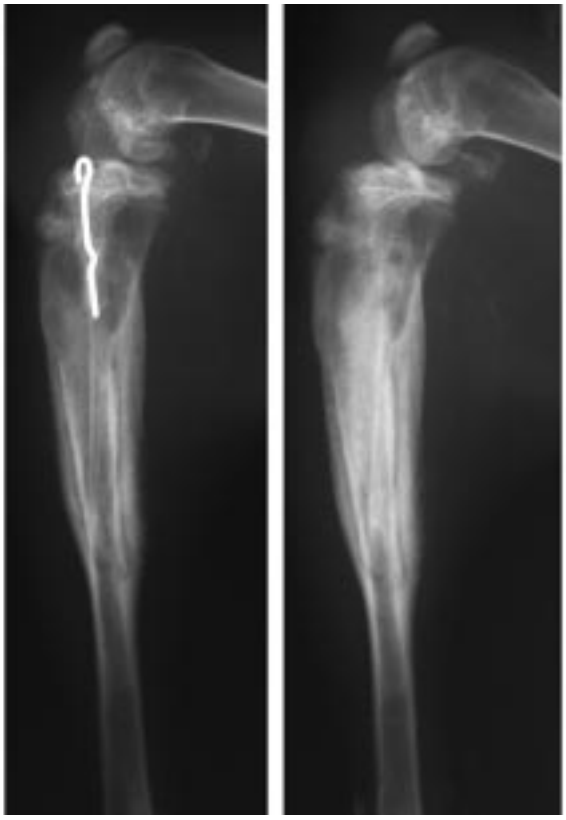


Figure 2. Lateral radiographs of the right tibia of the same rabbit at day-28, before (left) and after (right) revision surgery. Periostal reactive bone formation as a sign of local response to infection is seen in the proximal half of the tibia. At the time of revision surgery, the implant was removed and bone cement was injected into the tibial canal after debridement.

inserted cement (mean ± standard deviation) weighed 0.97±0.17 g in Group 1 (tobramycin cement), 1.03±0.13 in Group 2 (systemic antibiotic), and 1.02±0.16 g in Group 3 (control).

The development of an infection in all rabbits was confirmed at the time of revision by the presence of pus macroscopically and/or positive cultures of debrided tissue or the revised implant. The X-rays of all rabbits taken on day 28 all showed clear signs of reactive bone tissue predominantly at the proximal half of the right tibia, indicating a response to the presence of a local fulminant infection at the site of the implant (Figure 2).

No signs of side effects of systemic cefazolin, like diarrhea caused by pseudomembraneous colitis, were seen in rabbits treated with cefazolin.

No clear differences between the three treatment groups were seen in body temperature and loss of body weight, ESR and WBC. Table 1 shows the results of ESR and WBC.

Table 1: Erythrocyte sedimentation rate and white blood cell count

Group of rabbits	ESR (mm/hr, mean±SD)			
	Day 0	Day 7	Day 28	Day 42
Tobramycin	1.3±0.5	42.9±34.7	0.9±0.3	1.3±0.5
Cefazolin	1.1±0.4	35.3±27.2	1.0±0.0	1.1±0.4
Control	1.5±0.5	41.9±26.5	1.6±0.5	1.7±0.7
	WBC (x 10^9/l, mean±SD)			
	Day 0	Day 7	Day 28	Day 42
Tobramycin	5.5±1.6	13.2±3.5	4.7±0.7	6.7±1.7
Cefazolin	4.2±1.1	10.4±1.2	6.5±2.0	5.8±1.8
Control	4.3±0.7	10.8±1.9	5.8±2.4	6.8±2.3

ESR = erythrocyte sedimentation rate; WBC = white blood cell count

Bacteriological culture

The outcome of cultures is presented in Table 2. Results of culture of the cortex of the right tibia adjacent to the cement (weight 1.03 ± 0.03 g, mean ± SD) showed a decrease for both antibiotic groups (tobramycin-cement and systemic cefazolin) in comparison with the control group. Since in both antibiotic groups most rabbits (7 out of 9 and 8 out of 8, respectively)

Table 2. Outcome of cultures

Group of rabbits	Infection rate	Culture (mean ± SD, ^{10}log CFU/g)
Tobramycin	2/9	1.1 ± 2.2
Control	10/10	5.7 ± 1.4
Cefazolin	0/8	0

CFU/g = colony forming units per gram of bone

showed negative culture results, no statistical analysis was performed on the mean culture results to point out the difference. The rate of infection in both antibiotic groups was significantly lower than in the control group, $p<0.01$. The rate of infection in the tobramycin-cement group was slightly higher (2/9) but not significantly different from the systemic cefazolin group (0/8), $p = 0.47$. When the inoculum dose (10^5 or 10^6 CFU) was taken into account, the statistical analysis also showed significant differences between both antibiotic groups and the control group, $p < 0.01$, but no significant difference between the two antibiotic treatment groups, $p = 0.5$.

Reverse line blot hybridization assay

No PCR sample at 42 days follow-up was obtained in 6 out of the 27 rabbits of which culture results were available at that time. Figure 3 shows a detail of

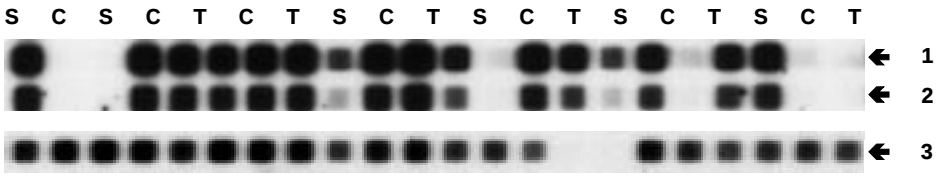


Figure 3. Details of the film with the results of the reverse line blot hybridization assay. PCR products of the right tibiae of the rabbits in the three different treatment groups are oriented in vertical lanes (T = tobramycin group, C = control group, S = systemic cefazolin group). The oligonucleotides are oriented in horizontal lanes (1 = staphylococci probe, 2 = *S. aureus* probe, 3 = internal spike probe). An internal spike was added to all samples to exclude possible inhibition: The lower lane shows no inhibition in samples that were negative for staphylococci or *S. aureus*

Table 3. Outcome of reverse line blot hybridization

	Culture -		Culture +	
	Tobramycin	2	Tobramycin	0
RLB -	Control	0	Control	3
	Cefazolin	1	Cefazolin	0
	Tobramycin	4	Tobramycin	1
RLB +	Control	0	Control	5
	Cefazolin	5	Cefazolin	0

The results (in number of rabbits) of the reverse line blot hybridization compared by culture. RLB = reverse line blot hybridization assay; - = negative result; + = positive result.

the film on which the results of the reverse line blot hybridization assay on samples (weight 0.34 ± 0.12 g, mean ± SD) of the right tibia of the other 21 rabbits are visualized. Table 3 relates the results of the reverse line blot hybridization assay on these samples to the subsequent culture results.

Discussion

In the present investigation, we demonstrated that both tobramycin-containing bone cement and systemic cefazolin reduce size and rate of infection in the treatment of an infected prosthesis by a one-stage revision procedure in rabbits, based on the results of bacteriological culture. This model was designed to mimic a one-stage revision procedure for an infected joint arthroplasty, so that a high efficacy of an antibiotic treatment would be required to clear the infection. We did not incorporate a two-stage revision model in this study, since our main objective was to test a specific antibiotic-containing cement. The temporary treatment between removal of the implant and insertion of the cement at a subsequent operation, may be a confounding factor for this purpose. Furthermore, this model differs from previous efficacy studies done by us and many other authors, in that the treatment did not start immediately after inoculation, but after a prolonged time period. This delay of treatment was introduced to create an established infection with the subse-

quent inflammatory responses and alterations in microcirculation and bone morphology. Therefore, in comparison with the 'direct-infection' models, this model is more similar to the clinical situation in which antibiotic treatment is started only after the infection has settled itself.

Only a few studies have addressed an animal model to evaluate the option of treating an established prosthesis-related infection with antibiotic-containing bone cement. Fitzgerald showed that gentamicin-containing bone cement could effectively prevent, but not treat *Staphylococcus aureus* infections around injected PMMA in tibiae of dogs [Fitzgerald, 1983]. In three out of five infections, the one-stage revision failed as a treatment. Gerhart *et al*. showed that gentamicin- and/or vancomycin- loaded bone cement had some, but no absolute efficacy in the treatment of *Staphylococcus aureus* infections in 34 rat tibiae, based upon the number of colony-forming units [Gerhart, 1993]. The number of failed treatments however, was not mentioned. The use of pre-formed cement to create an infection in this model could have made the removal of the cement easier as compared to the model of Fitzgerald, who inserted the initial cement in as dough.

In the present study, systemic cefazolin was effective in treating the infection in rabbits after revision of the implant. Salez-Mghir *et al*. studied the treatment with either systemic vancomycin or teicoplanin of a tibia implant infection in rabbits [Saleh Mghir, 1998]. Neither of these antibiotics could fully clear the infection in the majority of rabbits. In contrast with our study, these rabbits were inoculated with methicillin-resistant *S. aureus* and treated only for one week. Notably, also in contrast with our study, the implants in these rabbits were not revised before starting treatment, stressing the need for revising the prosthesis once infected. In present study, systemic antibiotic treatment was not significantly superior to local antibiotic treatment, although higher numbers of animals may have revealed such a difference. In another animal model, tobramycin-containing bone cement could prevent all infections [Nijhof, 2000b]. In the present study, the same cement could not fully treat 2 out of 9 infected rabbits. This can be explained by the more extensive spread of infection at the time of revision, i.e. not restricted only to the local area around the bone cement. Destruction of the cortex might be severe, and remaining necrotic tissue some distance from the antibiotic-containing bone cement may cause treatment failure [Fitzgerald, 1983]. In such a case, it is doubtful whether an antibiotic course of more than two weeks would have been more successful, although a previous study has shown that antibiotic elution from tobramycin-containing bone cement could be detected up to 4 weeks *in vivo* [Nijhof, 1997]. Clinically, in patients with such an

extensive implant infection, few surgeons will opt for only local antibiotics. For this matter, a combination of both antibiotic-containing bone cement and systemic antibiotic might be optimal: Systemic antibiotics for the wound problems and bacteria outside the operative area, and antibiotic-containing bone cement for local, high release of antibiotic. Indeed, this combination of antibiotic administration was predominantly used in many series reporting on one-stage revision, although an important early study of Buchholz *et al*. reported success rates of 77 to 90 per cent in one-stage revisions mainly without administration of systemic antibiotics [Buchholz, 1981, Loty, 1992, Miley, 1982, Mulcahy, 1996, Salvati, 1982a, Ure, 1998, von Foerster, 1991, Wroblewski, 1986].

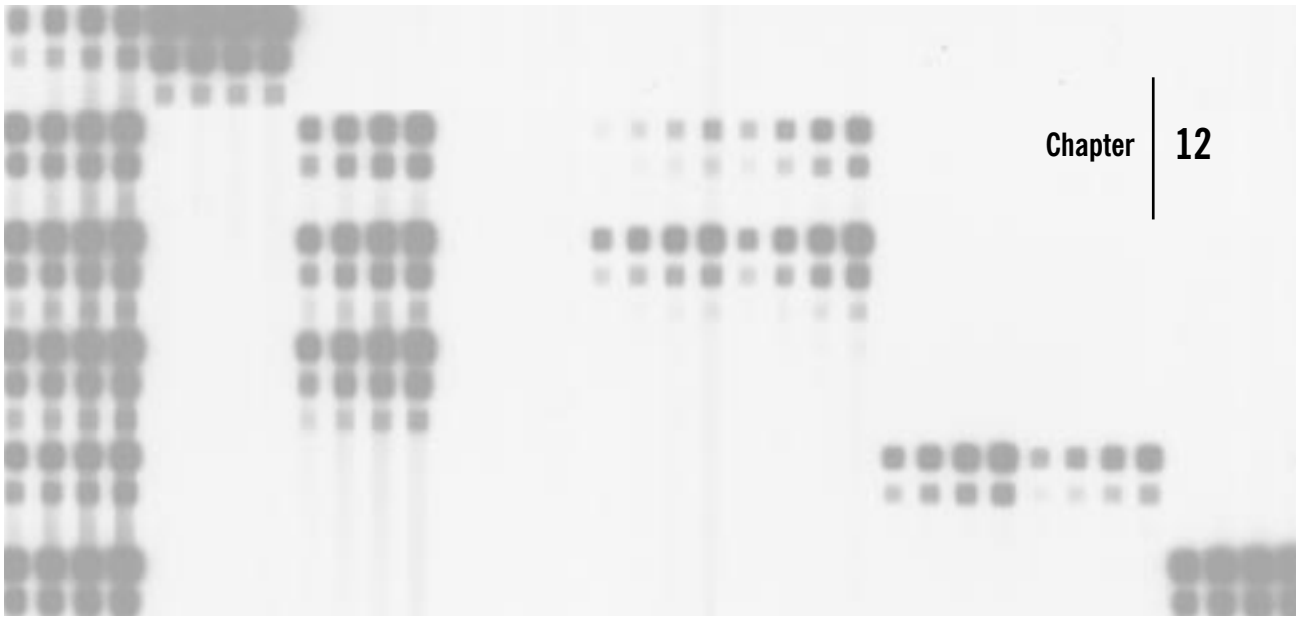
In addition to culture, we have determined the presence of staphylococcal DNA in the samples of the tibial cortex of the rabbits by means of a reverse line blot hybridization assay. PCR-based assays like RLB might become valuable complements of conventional microbiological techniques, or even improve diagnostic accuracy [Hoeffel, 1999, Mariani, 1996, Mariani, 1998, Tunney, 1999]. In 9 out of 12 rabbits with negative culture results, reverse line blot hybridization showed the presence of *S. aureus* DNA in the right tibia, 14 days after treatment with antibiotics (either systemically or with antibiotic-containing bone cement). Do these reverse line blot hybridization results demonstrate the sensitivity of DNA-based detection methods, or should these findings be interpreted as false-positive results based upon the outcome of culture? The sensitivity of a PCR-based detection method, using broad range bacterial primers, is at the same time its 'Achilles heel': PCR does not only amplify DNA of viable bacteria, present at the site of the implant, but also contaminating DNA or small quantities of DNA that can still be present shortly after antimicrobial killing of the bacteria. Strict policies in handling the samples should be and have been obtained in this study to exclude false-positive results due to contamination, either from contaminated reagents or from previously amplified bacterial DNA products. We think that the presence of *S. aureus* DNA as confirmed by the reverse line blot hybridization in rabbits with negative culture can be explained to a large extent by non-viable bacteria after antibiotic treatment. Clearance of all bacterial DNA after antibiotic treatment may be species specific, but van der Heijden *et al*. have shown that it can take up to 26 days after initiation of therapy that PCR of non-staphylococcal DNA becomes negative in septic joints [van der Heijden, 1999b]. In a previous animal model we studied the infection prophylaxis with systemic cefazolin or tobramycin-containing bone cement after inoculation of the rabbit tibia with *S. aureus* [Nijhof, 2000b]. *S. aureus* DNA

could not be detected 7 days after the procedure. These findings may implicate that the clearance of DNA after antibiotic treatment is dependent on bacterial load, since in the present model, the therapy was started only after a full-blown infection had developed. Further studies should be employed to address the persistence of bacterial DNA after antibiotic treatment, because DNA-based diagnosis will become increasingly important in the near future [Rantakokko-Jalava, 2000]. Important clinical decisions based on this type of diagnosis regarding the continuation of antibiotic therapy, whether systemically or locally via antibiotic-containing bone cement or beads, will benefit from more insight on this matter. Furthermore, the viability of microorganisms can be studied using molecular techniques targeting RNA. Reverse transcriptase PCR and nucleic acid sequence amplification (NASBA) have been used for this purpose [Sheridan, 1998, van der Vliet, 1994]. Both ribosomal RNA (rRNA) and messenger RNA (mRNA) can be targeted, but rRNA has been shown to persist longer than mRNA in mycobacteria after chemotherapy [Hellyer, 1999]. *In situ* hybridization for staphylococcal 16S rRNA can give additional information whether the detection of bacterial DNA represents the presence of the causative organism at the site of an implant or that it is caused by contamination [Krimmer, 1999].

RLB confirmed the presence of *S. aureus* DNA in only 6 of the 9 rabbits in which *S. aureus* was cultured 14 days after revision. Since we added an internal spike in the PCR samples, we could exclude inhibition of amplification in the 3 control rabbits with a negative reverse line blot hybridization result and a positive culture. These rabbits had a relatively low bacteria load as compared to the 6 rabbits with a positive reverse line blot hybridization result and positive cultures (respectively 3.8 ± 0.8 and 6.3 ± 0.6, mean $^{10}\log$ CFU/g). The PCR samples of the three rabbits with negative reverse line blot hybridization results may have originated from a part of the tibial cortex where infection was minimal, causing sampling error. Furthermore, the differences between the weights of the bone samples taken for culture or PCR, and the magnitude of dilution of the original volumes during the two techniques should be considered. The amount of a bone used for a PCR sample was approximately 500 times less than that for culture.

It is concluded from the current study that both tobramycin-containing bone cement and systemic cefazolin used in a one-stage revision for an infected implant can reduce size and rate of infection. However, in cases of virulent infections, a combination of systemic and local antibiotics may be necessary in a one-stage revision procedure. Further studies on the feasibility of PCR-reverse line blot hybridization are advised.

Summary and conclusions



Chapter 12

Osteomyelitis and other musculoskeletal infections have a major impact on the clinical outcome after trauma and surgical procedures. These infections can be the onset of a prolonged period of illness, hospitalization and disability of the patient. The treatment of such infections can be demanding for the (orthopaedic) surgeon when diagnostic and therapeutic options are limited. The financial costs involved with this complication certainly form an unwanted and high burden on health care and hospital budgets. The search for new techniques and products to optimize the prevention and treatment of musculoskeletal infections continues. A multifactorial approach that takes into account the variety of aspects involved in this clinical entity is required to achieve the goal of eliminating its development and consequences. This approach involves the identification of any risk factor that makes the patient prone to infection and adequate prophylactic measurements. Efficient techniques for detection and imaging of musculoskeletal infections facilitate early diagnosis and treatment. The (orthopaedic) surgeon will have to choose from a broad spectrum of products and techniques. Since options are multifold, the surgeon and other specialists involved should have knowledge about the performance of such techniques or products, so they can choose what is most appropriate for the specific patient and site and type of infection. Existing and new tools have to be evaluated in clinical trials, animal experiments and *in vitro* studies. This thesis describes studies on the applications of recently developed techniques for the imaging and detection of musculoskeletal infections in **Part I**, and a new premixed tobramycin-containing bone cement for the prevention and treatment of prosthesis-related infection in **Part II**.

Chapter 2 introduces the reader to the first part of this thesis on new tools for diagnosis of musculoskeletal infection. Firstly, several conventional and nuclear imaging techniques that are available in clinics or recently have been developed are being discussed in this chapter with regard to their use in musculoskeletal infection. Conventional imaging techniques like radiography, computed tomography and magnetic resonance imaging can depict anatomic alterations or water displacement caused by infection. The accuracy of visualization of osteomyelitis depends on time of imaging and possible alterations in anatomy after trauma, implant surgery or previous infection. Also, image interpretation of bone scintigraphy can be hampered in previously affected bone, because prolonged non-infectious uptake of the radiopharmaceutical technetium-99m-methylene diphosphonate designates the increased bone turnover in for instance fracture healing. Nuclear imaging modalities like ^{67}Ga-citrate scintigraphy and others that use radiolabeled leukocytes, antibodies, or liposomes show an uptake that is more functional rather than

anatomically. These techniques seem therefore more suitable for the visualization of the extent of musculoskeletal infection than the previously mentioned. Each scintigraphic imaging technique has its own specific advantages and disadvantages. These pros and cons depend among others on the mechanism of uptake of the tracer and the localization and nature of the infectious process. In the last few decades several radiopharmaceuticals have been developed in order to improve scintigraphic imaging of (musculoskeletal) infections. These scintigraphic agents should be investigated both pre-clinically and clinically for their performance in the imaging of different musculoskeletal infections at different sites in the body. These studies might provide better insight in the limitation of each technique, which enables the (orthopaedic) surgeon and nuclear medicine physician to choose on a more profound basis the most appropriate imaging technique.

The second part of the introduction in this chapter discusses the promises of the application of new molecular diagnostic techniques for the detection of infection in orthopaedic patients. Current available microbiological techniques like bacterial culture have shortcomings concerning speediness and sensitivity. Some bacterial species encountered in orthopaedics require a considerably prolonged culture time. In addition, some bacteria require specific growth conditions. Furthermore, other aspects of musculoskeletal infections can make them more difficult to culture. These include the fact that for the treatment of orthopaedic infections often multiple and prolonged courses of antibiotics are needed. Prior or concurrent antibiotic treatment may negatively influence the outcome of culture. Phenotypically reduced growth rates can also be found in bacteria embedded in biofilms on orthopaedic hardware like prostheses and fixators, or may be induced by local release of antibiotics from bone cement. New DNA based detection and identification methods have been developed, which show promising features that might strengthen our ability to detect bacterial infections. These tests can be run independently from specific growth requirements. These tools might also detect pathogens in a sensitive manner, since the number of copies of bacterial DNA can be amplified exponentially using the polymerase chain reaction technique.

The diagnosis of musculoskeletal infection will evolve when it can be shown that new techniques for detection and imaging appear to be useful tools for this indication. Eventually, some of the dilemmas that one can be confronted with every now and then in orthopaedics might be solved.

A study on the clinical use of a promising new agent for the imaging of infection and inflammation is presented in **Chapter 3**. A retrospective study on the performance of In-111-labeled human nonspecific IgG (In-111-IgG) was

performed involving over 200 patients with 232 possible foci of musculoskeletal infection or inflammation. After intravenous administration of a dose of 75 MBq In-111 labeled to 2 mg IgG (MacroScint), imaging was performed 4, 24, and 48 hours postinjection. The results were verified by culture, obtained either surgically (42%) or via puncture (19%), and long-term clinical and roentgenological follow-up (39%). Follow-up data were used in patients of whom the vast majority had a negative work-up, including negative In-111-IgG scintigraphy. All infected total hip (THA) and total knee (TKA) arthroplasties, focal osteomyelitis, diabetic foot infections, septic arthritis, and soft-tissue infections were detected (61 foci). Only one patient with early, low-grade spondylodiscitis was false-negative with In-111-IgG. Since In-111-IgG scintigraphy does not discriminate between infectious and sterile inflammation, careful interpretation is necessary in cementless THA up to one year after insertion. Uptake only around the neck of the femoral component of a THA, in recent fractures, and in pseudarthrosis are other pitfalls, since this may be caused by sterile inflammation. The overall specificity for infection of In-111-IgG scintigraphy is 77%. However, this study indicates that it might be possible to increase this specificity when the scintigrams are read in conjunction with radiographs and when particular patterns of increased uptake caused by sterile inflammatory processes are identified. It could be concluded that In-111-IgG scintigraphy is a very sensitive tool for detection of infectious bone and joint disease. Although uptake in inflammatory processes may decrease specificity of In-111-IgG scintigraphy for infection, specificity for most types of musculoskeletal infection was shown to be considerable.

In **Chapter 4** the performance of two new radiopharmaceuticals, Tc-99m-PEG-liposomes and Tc-99m-HYNIC-IgG, were evaluated in an experimental model of chronic osteomyelitis. For comparison, also Tc-99m-MDP, In-111-granulocytes and Ga-67-citrate were used. Chronic osteomyelitis was induced in rabbits by inserting *S. aureus* into the right reamed and washed femoral canal. The canal was closed with cement. A sham operation was performed on the left femur. Routine x-rays were obtained immediately after surgery and prior to scintigraphy. Four weeks after surgery, each rabbit was injected with 37 MBq Tc-99m-PEG-liposomes, Tc-99m-HYNIC-IgG and Tc-99m-MDP on three consecutive days, and imaged up to 4 (MDP) or 22 (liposomes and IgG) hours postinjection. On the fourth day, rabbit received either 18 MBq In-111-granulocytes or Ga-67-citrate and were imaged up to 44 hours postinjection. Uptake in the infected femur was determined by drawing regions of interest. Ratios of infected-to-sham-operated femur were calculated. After the

last image, the rabbits were killed and the left and right femur were scored for microbiological and histopathological evidence of osteomyelitis. The results showed that Tc-99m-PEG-liposomes and Tc-99m-HYNIC-IgG correctly identified all 6 rabbits with osteomyelitis. In-111-granulocytes and Ga-67-citrate gave equivocal results in one infected rabbit. Tc-99m-MDP missed one case of osteomyelitis. The uptake in the affected region did not differ significantly between the agents, although Tc-99m-MDP tended to have somewhat higher values (MDP 4.75 – 1.23 %ID, Ga-67 2.05 – 0.54 %ID, granulocytes 1.56 – 0.83 %ID, liposomes 1.75 – 0.76 %ID and IgG 1.96 – 0.27 %ID). The ratios of infected-to-normal femur were also not significantly different for the respective radiopharmaceuticals. Radiography only visualized severe osteomyelitis. It could be concluded that in this rabbit model, Tc-99m-PEG-liposomes and Tc-99m-HYNIC-IgG performed at least as well as In-111-granulocytes and Ga-67-citrate in the localization of chronic osteomyelitis. The ease of preparation, the better image quality and the lower radiation dose suggest that Tc-99m-PEG-liposomes and Tc-99m-HYNIC-IgG might be suitable alternatives for Ga-67-citrate and In-111-granulocytes in the scintigraphic evaluation of osteomyelitis.

In **Chapter 5**, a new DNA based technique for identification of bacterial pathogens of musculoskeletal infection was explored. This technique, reverse line blot hybridization (RLB), uses different oligonucleotide probes attached on a membrane to screen for reactivity on clinical specimens after a broad-range PCR on the 16S-rRNA gene, present in all bacteria. Species-, genus- and group-specific oligonucleotide probes were designed that were specific to bacteria frequently encountered in orthopaedic infections, based on information available from public database on the internet. An additional eubacterial probes screens for other, not specifically identified bacterial species in the specimen. The aim of this study was to design these oligonucleotide probes, and, subsequently, to test these for accuracy and clinical feasibility to detect bacterial PCR products. Amplified DNA from bacterial lysates and from patient material was used in this study. Correct hybridization signals were found for most probes, and preliminary tests in patients, mostly with implant related infections or septic arthritis, indicated promising results of the reverse line blot technique. Further studies are employed to improve design of some probes and to optimize hybridization conditions.

Improvement in diagnosis is only one feature involved in the optimization of patient care in musculoskeletal infection. The evaluation of techniques and products for use in prevention and treatment of musculoskeletal infection is an equally important approach for this matter. **Chapter 6** introduces **Part II**

of this thesis, in which a new tobramycin-containing bone cement is evaluated for its release of tobramycin *in vivo* and its efficacy in the prevention and treatment of prosthesis-related infection in rabbits. Since the introduction of gentamicin-containing bone cement in the late sixties by Buchholz and subsequent other combination of antibiotic and bone cements, the incidence of total hip prosthesis infection has decreased to a level below 1% and somewhat higher for infection of total knee prostheses. The high and increasing number of primary joint arthroplasties performed annually (e.g. approximately 17,000 total hip prostheses in The Netherlands and up to a million worldwide) makes that continuously effort is warranted to decrease this percentage. In numerous hospitals, especially in the United States, tobramycin powder is hand-mixed with bone cement for the preparation of antibiotic containing bone cement. The heat-stability of the aminoglycoside tobramycin favors its use to incorporate it in polymethylmethacrylate (PMMA). The antimicrobial spectrum of tobramycin includes pathogens like staphylococci and aerobic Gram-negative bacilli, which are most frequently cultured from orthopaedic implant infection. Figures in literature on the release of tobramycin from hand-mixed tobramycin bone cement and the efficacy of this cement in prosthesis infection underscore the applicability of tobramycin to add as the antibiotic in bone cement. However, these data are not derived from uniform studies in which only the same amount of tobramycin is mixed similarly in one type of bone cement. Pre-mixed tobramycin-containing bone cement might allow for a standardized preparation with a consistent and controllable quality of the cement. Such a product enables the comparison of future studies on its efficacy in orthopaedic infections and its biomechanical behavior, as well as its outcome in *in vitro*, animal or clinical studies in relation with other antibiotic-containing bone cements. The second part of this thesis reports from the first *in vivo* animal studies on the efficacy of this new pre-mixed tobramycin-containing bone cement in the prevention and treatment of prosthesis-related infections. This antibiotic-loaded carrier is one of many local drug delivery systems, available for the management of musculoskeletal infections. An overview is given in **Chapter 7**.

In the first of a series of studies on tobramycin-containing bone cement, tobramycin release was evaluated in a rabbit model. After insertion of cement into the right femur, tobramycin concentration as a function of time for up to 28 days was measured in serum and bone of rabbits. In **Chapter 8**, the tobramycin release in the femoral cortex adjacent to the cement was shown to be exceeding the minimal inhibitory concentration for *S. aureus* Wood 46. Serum tobramycin concentrations were below the systemic toxicity threshold.

The high local antibiotic levels in bone that can be achieved without compromising systemic safety is an important advantage of delivering antibiotic locally via bone cement. Such high tobramycin concentrations in bone might also be beneficial considering its concentration dependent antimicrobial killing, and might kill other pathogens that would be defined as resistant, based serum susceptibility tests. However, the capacity of bacteria to phenotypically alter their growth characteristics with subsequent decreased susceptibility to antimicrobial agents can counterbalance these benefits of antibiotic bone cement derived antibiotics like tobramycin. Animal and clinical studies are therefore necessary to assess the value of the use of this new pre-mixed tobramycin-containing bone cement in orthopaedic implant-related infection.

To perform such an animal study, we evaluated in **Chapter 9** the efficacy in a rabbit model on the performance of the pre-mixed tobramycin-containing bone cement to prevent prosthesis-related osteomyelitis. After intramedullary injection with staphylococci, either standard or tobramycin-containing Simplex-P bone cement was injected in the right femur of 120 rabbits. Development of infection was examined by culture of femoral bone after 7 or 28 days. Loss of body weight and elevated erythrocyte sedimentation rate in the control rabbits inoculated with *S. aureus* were seen in the first post-operative week, returning to normal in 28 days. Inoculation with *S. epidermidis* only resulted in a low-grade infection. All rabbits receiving tobramycin-containing bone cement were free of signs of infection and all their cultures were negative. Culture yield from *S. aureus* controls increased with time and inoculum dose. *S. epidermidis* controls needed higher inoculum doses to establish an infection, whereas culture yield decreased in time. The results of the study presented in this chapter showed that pre-mixed tobramycin-containing bone cement could prevent in this rabbit model osteomyelitis caused by two representatives of frequently encountered pathogens in orthopaedic implant-related infection. Pre-clinical trials will be needed to ensure that these results will be translatable to the use of this cement in human. Further studies are also needed to see whether the use of this or other antibiotic-containing bone cements are indicated and cost-effective in the prevention of prosthesis infections, especially in primary arthroplasties.

Currently, a much-preferred mode of antibiotic prophylaxis for orthopaedic implant surgery is the administration of systemic antibiotics. Cephalosporins are used widely for this indication. The goal of **Chapter 10** was to see whether systemic cefazolin could demonstrate a similar efficacy as compared to pre-mixed tobramycin-containing bone cement in the previously presented prevention model using a high inoculum dose. For this study, we used *S.*

aureus in a dose of 10^6 colony-forming units to inoculate the femur in 18 rabbits prior to injection of bone cement. The first group of six rabbits received tobramycin-containing Simplex-P bone cement. Two other groups of six rabbits received plain Simplex-P bone cement. Preoperatively, in one of the two latter groups cefazolin was administered intravenously. The other group served as untreated controls. The rabbits were monitored for clinical signs of infection. At 7 days follow-up, the femora were harvested and cultures from the bone adjacent to the cement plug were quantified. The reverse line blot technique, as described in Chapter 5, was used as an additional control to evaluate the outcome of this study. Cultures from the rabbits which received antibiotic prophylaxis (either cefazolin systemically or tobramycin-containing bone cement) were all negative. In contrast, all rabbits in the untreated control group had positive cultures. These rabbits also had other signs of infection such as an elevated erythrocyte sedimentation rate and loss of body weight. Culture results were confirmed by the absence of bacterial DNA in the PCR hybridization assay. In conclusion, we found that both tobramycin-containing bone cement and systemic cefazolin are effective in preventing implant bed infection in rabbits up to 7 days after contamination with *S. aureus*.

Another major indication for the use of pre-mixed tobramycin-containing bone cement can be expected to be its use for the treatment of an already established osteomyelitis, e.g. after revision of an infected prosthesis. A revision arthroplasty can be performed in a one- or two-stage procedure. The latter involves an additional period in which the implant bed is allowed to recover from the infection under antibiotic treatment. Whereas the one-stage procedure certainly has some advantages like less morbidity and initial lower costs, a higher risk of re-infection may decrease its cost-effectiveness in the long term. Thus, the indications for a one-stage procedure have yet to be refined, but this procedure seems to be a good option for evaluating new antimicrobial treatments in animal studies. Not only may it require a higher treatment efficacy of the treatment under investigation, but it is also limited by fewer confounding factors when compared with the two-stage procedure. Therefore, a one-stage revision model was used in **Chapter 11** to study the efficacy of tobramycin-containing bone cement as compared to that of systemic cefazolin for treatment of an infected implant. In addition, the value of detecting bacterial DNA after antibiotic treatment was investigated. An implant was inserted into the right tibia of rabbits after inoculation with *S.aureus*. At 28 days, the implant was removed. Subsequently, either plain bone cement with or without systemic administration of cefazolin, or

tobramycin-containing bone cement was injected into the medullary canal. The tibiae were cultured fourteen days after revision (day 42) and showed a significant decrease in bacterial counts for both antibiotic groups compared with the control group (p†0.05). The rate of infection in the tobramycin-cement group was slightly higher (2/9) than in the cefazolin group (0/8) although the difference was not significant. Persistence of bacterial DNA after antibiotic treatment may be the result of delayed clearance of DNA and not a sign of active infection. This animal model shows that in a one-stage revision tobramycin-containing bone cement can reduce size and rate of infection, although systemic cefazolin may be more efficacious. Therefore, the use of antibiotic-containing bone cement combined with systemic antibiotic might provide optimal treatment.

The clinical entity of musculoskeletal infection cannot be approached unidirectionally. The consequence of the multifactorial aspect of osteomyelitis and related infections is that the problems it confronts the orthopaedic surgeon and his colleagues with should be tackled from different directions. An early recognition of the development and extent of musculoskeletal infection is vital to its outcome and the prevention of subsequent complications. New techniques and products aiming for improvements in diagnosis, prevention and treatment of musculoskeletal infections are directed to provide standardized efficacious tools available for patient care in this clinical setting. Because of the complex nature of this infectious disorder, single studies like those presented in this thesis will not be sufficient to prove the efficacy of the new developments in all its facets. Continuous efforts in the evaluation of such new tools and those that are already in clinical use will be needed. Thus, a decrease in incidence and consequences of musculoskeletal infections is and should be an ongoing strive for both clinicians and researches involved in this subject.

Samenvatting en conclusies

Osteomyelitis en andere infecties van het steun- en bewegingsapparaat kunnen het klinisch beloop na trauma en chirurgie in belangrijke mate be nvloeden. Deze infecties resulteren soms in een lange periode van ziek zijn, ziekenhuisopname en lichamelijk ongemak. Beperkte diagnostische en therapeutische mogelijkheden kunnen de behandeling van deze infecties tot een opgave maken voor de (orthopaedisch) chirurg. Daarbij komt dat deze complicatie ook een zware financi le last is voor de huidige gezondheidszorg en een aanslag vormt op het ziekenhuisbudget. Onderzoek naar nieuwe technieken en middelen om te komen tot optimalisatie van de preventie en behandeling van infecties van het steun- en bewegingsapparaat blijft noodzakelijk. Hierbij is een multifactori le aanpak vereist die rekening houdt met de verschillende aspecten van deze klinische entiteit. Deze benadering houdt onder andere in het identificeren van de verschillende risicofactoren die bepalend zijn voor het ontstaan van infectie en de daaruit volgende adequate prophylactische maatregelen. Effici nte opsporing- en afbeeldingstechnieken van genoemde infecties faciliteren een vroege diagnose en behandeling. De (orthopaedisch) chirurg zal moeten kiezen uit een breed scala aan producten en technieken om te komen tot een optimale behandelingsstrategie. Kennis van de mogelijkheden en effectiviteit van elk van deze draagt bij tot het vinden van de meest geschikte strategie voor de specifieke pati nt, lokalisatie en type van de infectie. Bestaande en nieuwe technieken dienen te worden ge - valueerd in klinische studies, dierexperimenten en in vitro studies. Dit proefschrift beschrijft studies naar de toepassing van recent ontwikkelde technieken voor het opsporen en afbeelden van infecties van het steun- en bewegingsapparaat in Deel I, en een nieuw tobramycine-houdend botcement voor de preventie en behandeling van prothese-gerelateerde infecties in Deel II.

Hoofdstuk 2 is een inleiding op het eerste deel van dit proefschrift over nieuwe technieken in de diagnostiek van infecties van het steun- en bewegingsapparaat. Verschillende conventionele en nucleaire technieken waarmee deze infecties kunnen worden afgebeeld worden hier besproken. Conventionele technieken, zoals radiografie, CT en MRI kunnen anatomische veranderingen of waterverplaatsing door infectie afbeelden. De accuratesse waarmee osteomyelitis kan worden afgebeeld hangt af van de duur van de infectie en de eventuele verstoorde anatomie ten gevolge van trauma, implantaat-chirurgie of eerdere infectie. Ook de interpretatie van skeletscintigrafie kan worden belemmerd indien het bot voorafgaand aan de infectie reeds aangetast was, omdat een toegenomen technetium-99m-methylene

diphosphonaat stapeling ten gevolge van een verhoogd botmetabolisme bijvoorbeeld ook wordt gezien in/na een niet-ge nfecteerde fractuur. Nucleaire technieken zoals 67Ga-citraat scintigrafie en radiogelabelde leukocyten, antilichamen, of liposomen laten een meer functioneel dan anatomische bepaalde verhoogde stapeling zien. Daarom is het met deze technieken, beter dan met de conventionele, mogelijk om de uitgebreidheid van infectieuze processen aan te tonen. Elke scintigrafische techniek heeft zijn eigen specifieke voor- en nadelen in deze, afhankelijk van het mechanisme van stapeling van de tracer, en van de lokalisatie en eigenschappen van de infectie. In de laatste decennia zijn er verschillende radiofarmaca ontwikkeld om te komen tot verbeterde diagnostiek van infecties. Deze radiofarmaca moeten zowel klinisch als pre-klinisch ge valueerd worden om te komen tot gefundeerde inzichten in de mogelijkheden en beperking van elke middel, op basis waarvan de (orthopaedisch) chirurg en nucleair geneeskundige de meest ge igende afbeeldingstechniek kunnen aanwenden.

Het tweede deel van de introductie in hoofdstuk 2 behandelt de toepassingsmogelijkheden van nieuwe moleculair diagnostische technieken voor het opsporen van orthopaedische infecties. Huidige conventionele technieken zoals het kweken van bacteri n zijn niet optimaal, met name wat betreft snelheid en sensitiviteit. De groei van sommige bacterie-species die genoemde infecties kunnen veroorzaken is langzaam of stelt bijzondere eisen aan de kweekcondities. Ook door andere factoren kan het moeilijk zijn de verwekker van orthopaedische infecties aan te tonen door middel van kweek, zoals het feit dat voor de behandeling van deze infecties vaak meerdere en langdurige antibioticakuren nodig blijken te zijn. Dit kan de uitkomst van latere kweken negatief be nvloeden. Een fenotypisch verminderde groeiactiviteit wordt ook gezien in bacteri n die zich hebben ingenesteld in een biofilm op orthopaedische prothesen of osteosynthesemateriaal, maar kan ook zijn ge nduceerd door lokaal afgifte van antibiotica uit botcement. Er zijn nieuwe DNA-technieken voor het aantonen en identificeren van bacteri le infecties beschikbaar die de diagnostiek in deze mogelijk kunnen verbeteren. Een belangrijk aspect hierbij is dat deze technieken niet afhankelijk zijn van specifieke groeicondities. Mogelijk kan de sensitiviteit van de diagnostiek ook toenemen omdat middels de polymerase ketting reactie eventueel aanwezig bacterieel DNA exponentieel kan toenemen.

De diagnostiek van infecties van het steun- en bewegingsapparaat zal verbeteren indien door middel van onderzoek kan worden aangetoond dat nieuwe detectie- en afbeeldingstechnieken hiervoor bruikbare instrumenten blijken te zijn.

In **Hoofdstuk 3** wordt een studie beschreven van een recent ontwikkeld radiofarmacon voor het scintigrafisch afbeelden infectie- en ontstekingshaarden. Hiertoe werd een retrospectieve studie verricht naar de performance van In-111-labeled human nonspecific IgG (In-111-IgG) in meer dan 200 pati nten met 232 mogelijke infectie- of ontstekingshaarden. Na intraveneuze toediening van 75 MBq In-111 gelabeld aan 2 mg IgG werden op 4, 12 en 48 uur na injectie opnames gemaakt. De resultaten hiervan werden geverifieerd aan de hand van kweekuitslagen van peroperatief (42%) of via punctie (19%) afgenomen weefsel, en door lange klinische en r ntgenologische follow-up (39%). Deze laatste gegevens werden gebruikt in pati nten waarvan in het merendeel geen infectie werd afgebeeld, ook niet middels In-111-IgG scintigrafie. Alle ge nfecteerde totale heup of knieprothesen, focale osteomyelitiden, diabetische voet infecties, septische arthritiden en weke delen infecties werden scintigrafisch afgebeeld. E n pati nt met een laaggradige spondylodiscitis had een vals-negatief resultaat na In-111-IgG scintigrafie. Het is belangrijk om de uitkomst van de In-111-IgG scintigrafie goed te interpreteren bij niet-gecementeerde heuparthroplastieken, vooral tot n jaar na plaatsing, omdat met deze techniek discriminatie tussen infectie en ontsteking in principe niet mogelijk is. Verhoogde stapeling rond de hals van een totale heupprothese, in recente fracturen en pseudarthrosen is een andere valkuil, omdat dit ook veroorzaakt kan zijn door steriele ontsteking. De specificiteit van In-111-IgG scintigrafie is 77% voor de totale groep pati nten. Deze studie wijst echter aan dat het mogelijk is deze specificiteit te verhogen wanneer de scintigrafische afbeeldingen wordt gelezen in samenhang met de r ntgenologische en wanneer bepaalde patronen van verhoogde stapeling worden ge dentificeerd als specifiek voor steriele ontsteking. Geconcludeerd kon worden dat In-111-scintigrafie een sensitieve techniek is voor het opsporen van bot- en gewrichtsinfecties. Hoewel stapeling in inflammatoire processen de specificiteit van In-111-IgG voor infectie kan verlagen, was de specificiteit voor de meest typen van infecties van het steun- en bewegingsapparaat aanzienlijk.

In **Hoofdstuk 4** worden twee nieuwe radiofarmaca, te weten Tc-99m-PEG-liposomen and Tc-99m-HYNIC-IgG, ge valueerd in een experimenteel model van chronische osteomyelitis. Ter vergelijking werden ook Tc-99m-MDP, In-111-granulocyten en Ga-67-citraat gebruikt. Chronische osteomyelitis werd opgewekt in konijnen door S. aureus te injecteren in de mergholte van het rechter femur, en deze holte te sluiten met cement. Het linker femur werd gebruikt als controle (geen inoculatie). Standaard r ntgenfoto s werden gemaakt aansluitend aan de operatie en voorafgaand aan de scintigrafie

opnames. Vier weken na de operatie kreeg elk konijn 37 MBq Tc-99m-PEG-liposomen, Tc-99m-HYNIC-IgG en Tc-99m-MDP toegediend op drie achtereenvolgende dagen, waarna afbeeldingen werden vervaardigd tot 44 uur na de injectie van de radiofarmaca. Stapeling in het ge nfecteerde femur werd vergeleken met die in het controle femur. Na de laatste opname werden de konijnen gedood en beide femora gescoord op microbiologische en histologische tekenen van osteomyelitis. Tc-99m-PEG-liposomen en Tc-99m-HYNIC-IgG identificeerden alle zes konijnen met osteomyelitis correct. De afbeeldingen vervaardigd met In-111-granulocyten en Ga-67-citraat waren moeilijk te interpreteren in n ge nfecteerd konijn. Tc-99m-MDP miste n geval van osteomyelitis. De stapeling in het aangedane gebied verschilde niet significant tussen de radiofarmaca, hoewel Tc-99m-MDP meestal wat hogere opname liet zien (MDP 4,75 – 1,23 %ID, Ga-67 2,05 – 0,54 %ID, granulocyten 1,56 – 0,83 %ID, liposomen 1,75 – 0,76 %ID en IgG 1,96 – 0,27 %ID). De ratio s van opname in ge nfecteerde femora ten opzichte van controles verschilden ook niet significant voor de respectievelijke radiofarmaca. Alleen ernstige infecties waren zichtbaar op r ntgenfoto s. Geconcludeerd werd dat in dit konijnenmodel Tc-99m-PEG-liposomen en Tc-99m-HYNIC-IgG op zijn minst net zo goed presteerden als In-111-granulocyten en Ga-67-citraat in het localiseren van chronische osteomyelitis. Het gemak van de preparatie van het radiofarmacon, de betere afbeeldingskwaliteit en de lagere stralingsdosis suggereren dat Tc-99m-PEG-liposomen en Tc-99m-HYNIC-IgG goede alternatieven zijn voor Ga-67-citraat en In-111-granulocyten om osteomyelitis scintigrafisch te evalueren.

In **Hoofdstuk 5** wordt een nieuwe DNA-techniek voor het identificeren van bacteri le pathogenen van infecties van het steun- en bewegingsapparaat beschreven. Deze techniek, de zogenaamde reverse line blot hybridisatie (RLB), maakt gebruikt van verschillende oligonucleotide probes die gekoppeld zijn aan een membraan. Eventuele reactiviteit met geamplificeerd DNA, na een algemene 16S PCR gericht op het 16S-rRNA gen dat aanwezig is in alle bacteri n, wordt hiermee getest. Species- genus- en groep-specifieke oligonucleotide probes, gericht op veel voorkomende bacteri n in orthopaedische infecties, werden ontworpen. Hiertoe werd gebruik gemaakt van informatie uit publieke databases op het internet. Een extra eubacteri le probe screent ook op andere, niet specifiek ge dentificeerde bacteri n in het preparaat. Het doel van deze studie was het testen op accuratesse van deze techniek en de mogelijkheid om dit toe te passen op klinische monsters. Geamplificeerd DNA van bacteri le lysaten en van pati nten monsters werden hiertoe gebruikt. De meest probes gaven een juist hybridisatie signaal en

eerste klinische resultaten in pati nten met vooral implantaat-gerelateerde infecties of septische arthritis zijn veelbelovend. Nadere studies worden opgezet om te komen tot een verbeterd ontwerp van sommige probes en een optimalisatie van hybridisatie condities.

Verbetering van de mogelijkheden tot het stellen van de diagnose is slechts n aspect van het optimaliseren van de zorg voor de pati nt met een infectie van het steun- of bewegingsapparaat. Net zo belangrijk is het evalueren van technieken en producten gebruikt in de preventie en behandeling van deze infecties.

Hoofdstuk 6 introduceert Deel II van dit proefschrift, waarin een nieuw tobramycine-houdend botcement wordt ge valueerd op de afgifte van tobramycine in vivo en zijn werkzaamheid in de preventie en behandeling van prothese-gerelateerde infecties in konijnen. Sinds de invoering van gentamicine-houdend botcement eind jaren zestig (Buchholz) en andere antibioticum-botcement combinaties, is de incidentie van totale heupprothese infecties gedaald tot onder de 1%. Voor totale knieprothese infecties ligt de incidentie iets hoger. Het grote en toenemende aantal primaire heuparthroplastieken dat jaarlijks wordt uitgevoerd (jaarlijks ongeveer 17.000 totale heupprotheses in Nederland en tot een miljoen wereldwijd) noopt tot een continue aandacht voor het terugdringen van infecties. In een groot aantal ziekenhuizen, met name in de V.S., wordt tobramycine-poeder met de hand vermengd met botcement om een antibioticum-houdend botcement te maken. Het aminoglycoside tobramycine is hittebestendig, waardoor het geschikt is om te gebruiken in polymethylmethacrylaat (PMMA). Het antimicrobi le spectrum van tobramycine omvat pathogenen als staphylococcen en aerobe Gram-negatieve bacillen, welke de meest frequente verwekkers zijn van orthopaedische implantaat infecties. Cijfers in de literatuur over de afgifte van tobramycine uit handmatig gemengd tobramycine-houdend botcement en de werkzaamheid van dit cement in prothese infecties onderschrijven de toepassingsmogelijkheid van het toevoegen van tobramycine in botcement. Deze cijfers komen echter niet van uniforme studies waarin een gelijke hoeveelheid tobramycine is vermengd met n type botcement. Een gestandaardiseerd product van een consistente kwaliteit zou kunnen worden verkregen door een voor-vermengd tobramycine-houdend cement van een constante en controleerbare kwaliteit. Zo n product laat vergelijkingen toe van toekomstige studies naar de werkzaamheid van dit cement in orthopaedische infecties en zijn biomechanische gedrag, en bovendien van uitkomsten uit in vitro, dier- of klinische studies in relatie tot andere antibioticum-botcement combinaties. Deel II van dit proefschrift beschrijft de

eerste in vivo dierexperimenten naar de werkzaamheid van dit nieuwe voorvermengde tobramycine-houdend botcement in de preventie en behandeling van prothese-gerelateerde infecties.
Antibioticum-houdend botcement is een van de vele systemen die voor de behandeling van infecties van het steun- en bewegingsapparaat beschikbaar zijn, waarmee lokaal antimicrobi le middelen kunnen worden afgegeven. **Hoofdstuk 7** geeft hiervan een overzicht.
In de eerste van een serie studies omtrent tobramycine-houdend botcement werd de afgifte van tobramycine ge valueerd in een diermodel. Na het inspuiten van tobramycine-houdend botcement werd de tobramycineconcentratie als functie van tijd tot en met 28 dagen gemeten in serum en bot.

Hoofdstuk 8 laat zien dat de afgifte van tobramycine in de cortex van het femur de MIC waarde voor S. aureus Wood 46 overstijgt. De tobramycineconcentraties in het serum lagen onder de systemische toxiciteitsdrempel. De hoge locale antibioticum-concentratie die kan worden bereikt in bot, zonder de systemische toxiciteitsdrempel te overschrijden, vormt een belangrijk voordeel van de locale toediening van antibiotica via botcement. Zulke hoge tobramycine-concentraties in het bot kunnen ook een gunstig effect hebben op de antimicrobi le werking, aangezien deze dosis afhankelijk is. Mogelijk is het antibioticum dan ook werkzaam tegen resistente (gebaseerd op serumconcentraties) bacteri n. Echter, het vermogen van bacteri n om fenotypisch hun groei aan te passen, resulterend in een verlaagde gevoeligheid antibiotica, kan de voordelen van het antibioticum-houdend botcement tenietdoen. Klinische studies en dierexperimenten zijn daarom nodig om de waarde van het gebruik van het nieuwe voor-vermengde tobramycine-houdende botcement in orthopedische, implantaatgerelateerde infecties te bepalen.
Door middel van dierexperimenteel onderzoek is in **Hoofdstuk 9** de werking van het nieuwe tobramycine-houdend botcement ten aanzien van prothesegerelateerde osteomyelitis ge valueerd. Na intramedullaire injectie met stafylococcen, werd zowel standaard botcement als tobramycine-houdend Simplex-P botcement ge njecteerd in de rechter femur van 120 konijnen. Na zeven of 28 dagen werd door middel van kweken onderzocht of er een infectie was ontstaan in het femur-bot. In de eerste week na de operatie werd in de S. aureus controlegroep gewichtsverlies en een verhoogde bezinkingssnelheid van erythrocyten gemeten. Na 28 dagen was de toestand bij deze groep weer normaal. Inoculatie met S. epidermidis leidde slechts tot een geringe infectie. Geen van de konijnen die tobramycine-houdend botcement kregen, toonde tekenen van infectie. Ook hun kweken bleven negatief. Kweken van de S. aureus controlegroep namen toe in de tijd en waren afhankelijk van de

toegediende dosis. Konijnen uit de S. epidermidis groep hadden hogere doses nodig om een infectie tot stand te brengen en kweken van deze groep namen af in de tijd. De resultaten in **hoofdstuk 9** laten zien dat tobramycine-houdend botcement in dit diermodel osteomyelitis kan voorkomen wanneer sprake is van S. aureus of S. epidermidis, ziektekiemen die regelmatig voorkomen bij implantaat-gerelateerde infecties. Toekomstige studies moeten aantonen of deze resultaten ook gelden bij gebruik van dit cement in de kliniek. Nadere evaluatie is ook gewenst om na te gaan of antibioticum-houdend botcement doeltreffend en kosteneffectief is ten aanzien van de preventie van prothese-infecties, met name bij primaire arthroplastieken.

Een veelgebruikte methode van infectiepreventie bij orthopedische implantaatchirurgie is de toediening van systemische antibiotica, zoals cefalosporinen. Het doel van de studie in **Hoofdstuk 10** was na te gaan of systemisch cefazolin, eenzelfde effect ten toon kon spreiden als tobramycine-houdend botcement in het eerder gepresenteerde diermodel, wanneer een hoge inoculum dosis (S. aureus, 106 cfu) werd gebruikt. S. aureus werd voorafgaand aan de toediening van cement ingebracht in het femur van 18 konijnen. De eerste groep van zes konijnen kreeg tobramycine-houdend Simplex P-botcement. Twee andere groepen van zes konijnen kregen gewoon Simplex-P botcement. Pre-operatief werd in een van de twee laatste groepen cefazolin intraveneus toegediend. De andere groep gold als onbehandelde controlegroep. De konijnen werden onderzocht op klinische verschijnselen van infectie. Na zeven dagen werden de femora verwijderd en de cortex rond de cementplug gekweekt, waarna de groei werd gekwantificeerd. De reverse line blot techniek, zoals beschreven in hoofdstuk 5, werd ingezet als extra controlemiddel bij de evaluatie van de resultaten van deze studie. De kweken van konijnen die antibiotische profylaxe kregen (systemisch cefazolin of tobramycine-houdend botcement), waren allemaal negatief. Daarentegen waren de kweken van alle konijnen uit de onbehandelde groep positief. Deze konijnen vertoonden ook andere tekenen van infectie, zoals gewichtsverlies en een verhoogde bezinkingssnelheid van de erythrocyten. De kweekresultaten werden bevestigd door de afwezigheid van DNA in de PCR-hybridisatie assay. Dus zowel tobramycine-houdend botcement als systemisch cefazolin is effectief in het voorkomen van implantaatbed infecties in konijnen tot zeven dagen na besmetting met S. aureus.

Een andere belangrijke indicatie voor het gebruik van tobramycine-houdend botcement kan een reeds ontstane osteomyelitis zijn, bijvoorbeeld na revisie van een ge nfecteerde prothese. Een prothese kan gereviseerd worden in een of twee fases. Een revisie in twee fases omvat een extra periode waarin het

implantaatbed de tijd krijgt om te herstellen van de infectie door behandeling met antibiotica. Hoewel directe revisie duidelijke voordelen heeft, zoals een lagere morbiditeit en lagere kosten op de korte termijn, is er een hogere kans op hernieuwde infectie en daarmee hogere kosten op de langere termijn. Indicaties voor deze procedure moeten dus worden verfijnd. Deze techniek, toegepast in een proefdiermodel, lijkt een goede optie om nieuwe antimicrobi le behandelingen te bestuderen. Dit zou een goede werkzaamheid van de te onderzoeken behandeling vergen. Een voordeel is dat het model, vergeleken met een revisie in twee fasen, minder wordt belemmerd door storende variabelen. Daarom is in **Hoofdstuk 11** een revisie in een fase gekozen als opzet van een studie naar de effectiviteit van tobramycine-houdend botcement in vergelijking tot systemische cefazolin bij de behandeling van ge nfecteerde implantaten. Tevens werd de waarde van de detectie van bacterieel DNA na behandeling met antibiotica onderzocht. In de rechter tibia van konijnen werd, na inoculatie met S. aureus, een implantaat ingebracht. Na 28 dagen werd het implantaat verwijderd. Vervolgens werd zowel normaal botcement, met of zonder systemische toediening van cefazolin, of tobramycine-houdend botcement ingebracht in de mergholte. De tibiae werden veertien dagen na de revisie (dag 42) gekweekt. De kweken toonden voor beide antibiotische groepen een significante afname in bacteriegroei vergeleken met de controlegroep ($p<0,05$). De mate van infectie in de tobramycine-cement groep was enigszins, maar niet significant, hoger (2/9) dan in de cefazolin groep (0/8). De aanwezigheid van bacterieel DNA na behandeling met antibiotica zou kunnen duiden op een vertraagde opruiming van DNA in plaats van op een actieve infectie. Dit diermodel laat zien dat tobramycine-houdend botcement de grootte en mate van infectie kan reduceren na een revisie in n fase, hoewel systemisch cefazolin mogelijk effectiever is. Het gebruik van antibioticumhoudend botcement gecombineerd met systemische antibiotica zou mogelijk aangewezen kunnen zijn indien een virulente infectie wordt behandeld met deze techniek.

De klinische entiteit van infecties van het steun- en bewegingsapparaat kan niet eenzijdig worden benaderd. Omdat osteomyelitis en gerelateerde infecties multifactorieel zijn, moeten de problemen die het schept voor de orthopaedisch chirurg en zijn collegae vanuit verschillende invalshoeken worden benaderd. Een vroegtijdige herkenning van de ontwikkeling en de uitbreiding van een infectie van het steun- en bewegingsapparaat is van cruciaal belang voor het verloop van de infectie en ter voorkoming van eventuele complicaties. Nieuwe middelen en technieken gericht op het verbeteren van

de diagnose, preventie en behandeling van infecties van het steun- en bewegingsapparaat worden ontwikkeld om te voorzien in gestandaardiseerde doeltreffende instrumenten voor de zorg van pati nten met deze aandoening. Vanwege de complexe aard van deze infecties zijn de afzonderlijke studies, zoals beschreven in dit proefschrift, niet afdoende om de doeltreffendheid van nieuwe ontwikkelingen in alle facetten te bewijzen. De evaluatie van dergelijke nieuwe middelen vergt continu nader onderzoek. Een afname van het aantal infecties van het steun- en bewegingsapparaat en de gevolgen daarvan is en zal dan ook een voortdurend streven moeten zijn van eenieder betrokken bij dit onderwerp.

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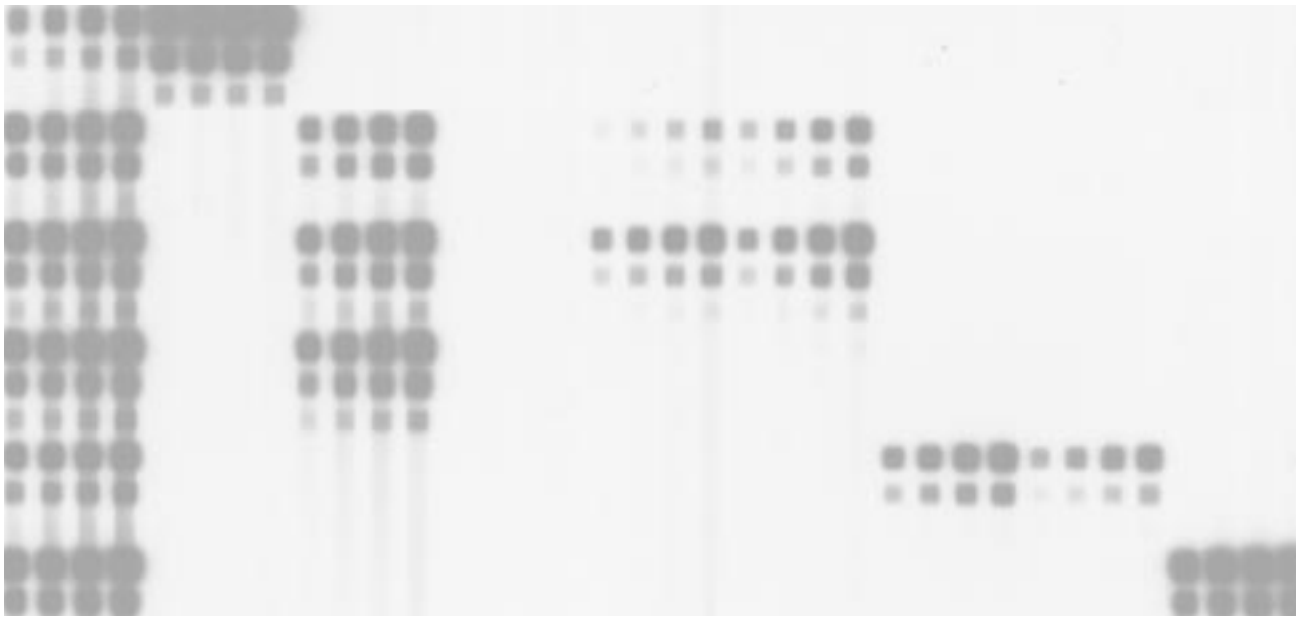

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Curriculum Vitae

Marc Willibrordus Nijhof was born on November 16, 1969, in Borne, The Netherlands. After graduating from high school (RK Lyceum 'De Grundel', Hengelo) in 1988, he studied medicine at the Katholieke Universiteit Nijmegen (1988-1995). In 1996 he worked some months as a resident at the department of Nuclear Medicine, University Hospital Nijmegen St Radboud (head: Prof. dr. F.H.M. Corstens), where also a part of the research for this thesis was performed. From 1996 to 2000 he performed his PhD research at the department of Orthopaedics, University Medical Center Utrecht. (Head: Prof. dr. A.J. Verbout). This project included studies performed at the Research Laboratory for Infectious diseases (LIO) of the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands (Head: dr. T.G. Timman) and at the Eijkman-Winkler Institute, University Medical Center Utrecht (head: Prof. Dr. J. Verhoef)

In 2001, he started a two-year residency in general surgery at the Medisch Spectrum Twente , Enschede, The Netherlands (head: Prof. Dr. P.A.M. Vierhout) and will start his training in orthopaedic surgery at the University Medical Center Utrecht thereafter.