In vivo comparison of Dhvar-5 and gentamicin in an MRSA osteomyelitis prevention model

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Objectives: The continued rise in drug-resistant pathogens has led to global research efforts into new antimicrobial agents. A promising class of new agents are the antimicrobial peptides. The aim of the study was to investigate the efficacy of the antimicrobial peptide Dhvar-5 in a prophylactic, methicillin-resistant Staphylococcus aureus (MRSA) osteomyelitis model.

Methods: Dhvar-5 (12 mg or 24 mg/rabbit) was incorporated into polymethyl methacrylate (PMMA) beads as a local drug delivery system. For comparison, plain beads (control) and beads containing gentamicin as a sulphate (10 mg or 24 mg per rabbit) were also prepared. The beads were inserted into the inoculated femoral cavity of 36 rabbits, and 1 week later they were killed. The presence and severity of MRSA osteomyelitis was assessed by culture and histology.

Results: Both the 24 mg Dhvar-5 beads and the 24 mg gentamicin sulphate beads significantly reduced the bacterial load of the inoculated femora compared with the control chain. Although a 24 mg Dhvar-5 dose inhibited MRSA growth, it did not completely sterilize the femora. Sterilization occurred only in some of the gentamicin-treated specimens.

Conclusion: We conclude that both the gentamicin beads and the Dhvar-5 beads were only partially effective at preventing MRSA infection in this model.

Keywords: antimicrobial peptides, drug delivery, rabbits, PMMA beads

Introduction

Osteomyelitis causes major morbidity and remains a difficult complication to treat in orthopaedic surgery. Radical surgical debridement with local administration of antibiotics, e.g. gentamicin-loaded polymethyl methacrylate (PMMA) beads, in combination with systemic antibiotics is the treatment of choice. The long-term release profiles of gentamicin from PMMA beads have been demonstrated in kinetic release studies. Overall, the use of gentamicin-loaded PMMA beads results in high local antibiotic bone and soft tissue concentrations versus low systemic concentrations. However, recent studies have shown an increase in antibiotic-resistant bacteria such as gentamicin-resistant Staphylococcus aureus (GRSA) and methicillin-resistant S. aureus (MRSA). Prevalence of MRSA in nosocomial infections has exceeded 30% in many countries, e.g. southern Europe and the USA. Neut et al. demonstrated growth of resistant staphylococci on retrieved gentamicin-loaded PMMA beads, raising concern about the efficacy of this treatment option. Furthermore, first reports of S. aureus vancomycin resistance have now been published. As current antibiotic therapy options for staphylococcal infections are becoming limited, there is an urgent need for new antimicrobial agents to combat these resistant pathogens.

Antimicrobial peptides (AMPs) are a new and promising class of antibiotics, derived from naturally occurring peptides. These peptides are found on epithelial surfaces, in secretion fluids and in neutrophils, and thus form a first line of host defence as part of the innate immune system. AMPs have generated a lot of interest as possible new antimicrobial...
agents; van’t Hof et al.5 have reviewed several AMPs under investigation. An advantage is that they have a diminished tendency to induce resistance because of the evolutionary difficulty in changing bacterial membrane structure.7,8

Dhvar-5 is an experimental AMP based on histatin-5, which is an antifungal peptide found in human saliva.10 Dhvar-5 has been designed to create a net positive charge at the C-terminus and a hydrophobic N-terminus (LLLFLKKRRKKRKY).10 The positive charge is presumed to play a crucial part in its antimicrobial activity.11 It disrupts and penetrates the negatively charged bacterial cell wall, and after cellular entrance it is targeted to intracellular organelles.12 The Dhvar-5 peptide has fungicidal and bactericidal activity and has antimicrobial activity against MRSA in vitro.13,14

In the light of antimicrobial resistance and the urgent need for new antimicrobial agents, we previously investigated the suitability of PMMA mini-beads as a carrier of Dhvar-5.15 These beads showed a high and prolonged in vitro release of Dhvar-5 in its biologically active form.15 Therefore, the aim of the present investigation was to evaluate the efficacy of Dhvar-5 and gentamicin beads in an in vivo MRSA osteomyelitis prevention model.

Materials and methods

Experimental design

The femoral cavity of 36 rabbits was inoculated with approximately 107 colony-forming units (cfu) of MRSA in a modified prophylactic osteomyelitis model described by Nijhof et al.16 Immediately after inoculation, a custom-made PMMA mini-bead chain was inserted into the femur. The chains were prepared as described previously;15 the control group (n=8) received a chain of 10 plain cement beads (Osteopal; Biomet Merck, Darmstadt, Germany) without antibiotic. The AMP-treated animals received a chain of 10 beads containing a total of 12 mg (n=7) and 24 mg (n=9) of Dhvar-5. The gentamicin-treated animals received a chain prepared from Osteopal G (Biomet Merck) containing a total of either 10 mg (n=7) or 24 mg (n=5) of gentamicin sulphate. Experimental groups were augmented to contain at least seven animals for statistical purposes, except for the 12 mg Dhvar-5 group and the 24 mg gentamicin sulphate group. After 7 days, the animals were killed and the femoral cortex was cultured for the presence of MRSA and the number of cfu per gram of bone tissue was determined as a measure for treatment outcome.

Animals

Female New Zealand White (NZW) rabbits (3.5–4 kg) were obtained 3 weeks before surgery and allowed to acclimatize to the Clinical Animal Laboratory, Vrije Universiteit Medical Center (VUmc). The animals were housed in groups and allowed water and antibiotic-free rabbit diet ad libitum. The experimental protocol was approved by the Animal Ethics Committee of our institution.

Preparation of PMMA mini-beads

The bead chains were prepared as described previously.15 In short, two different quantities of Dhvar-5 (50 mg and 100 mg/g of cement powder) were mixed with the cement powder. The liquid monomer was added in a 1:2 liquid to powder ratio and the resulting cement paste was injected into a custom-made mould. The cement was allowed to polymerize on a stainless-steel wire (0.3 mm diameter), removed from the mould and stored inside a sealed tube at −20°C until use. This process produced a chain of 10 beads containing a total of either 12 or 24 mg of Dhvar-5. The 10 mg gentamicin sulphate chains were obtained by injecting the commercially available Osteopal G (Biomet Merck) bone cement into the mould. According to the manufacturer, Osteopal G is identical to Osteopal except for the gentamicin content. The gentamicin sulphate content of the Osteopal G cement powder was increased from 41.75 to 100 mg gentamicin sulphate per gram cement powder to produce the 24 mg gentamicin sulphate chains. From here on, the group dosages referred to as gentamicin are the gentamicin sulphate dosages. As each animal received one chain of 10 beads during surgery, the dosages mentioned are the amount of antibiotics incorporated into a chain of 10 beads, and therefore the amount per animal.

Bacterial strain

The MRSA strain (ATCC: BAA-811) was isolated from an osteomyelitis patient and was resistant to several antibiotics including gentamicin (Table 1). An overnight culture was grown in brain heart infusion (BHI) broth and washed in PBS. Subsequently, aliquots were prepared and stored at −80°C. The amounts of viable cfu were determined in defrosted aliquots by serial dilution and plating on blood agar. On the day of surgery a fresh aliquot was allowed to thaw and a bacterial suspension of 106 cfu/mL was prepared in PBS. The femoral canal was inoculated by a direct injection of 0.1 mL of bacterial suspension (107 cfu). After surgery the inoculum was serially diluted and plated on blood agar for determination of viable cfu count.

Anaesthesia and surgery

The animals were weighed and subcutaneously injected with xylazine 2.5 mg/kg (Xylalin; CEVA Sante Animale, Maassluis, The Netherlands) and ketamine 62.5 mg/kg (Aescoket; Aesculaap, Boxtel, The Netherlands). Once sedated, the right hip area was shaved and painted with iodine. Subsequently, lidocaine 5 mg/kg (Pharmacy VUmC, Amsterdam, The Netherlands) was injected around the greater trochanter. Preoperative blood samples were taken from the right auricular vein for the determination of peripheral white blood cell count (WBC) and erythrocyte sedimentation rate (ESR). The animals were placed on their left side and the skin was again painted with iodine and the surgical area was isolated with sterile drapes. During surgery, the anaesthesia was maintained by free flow inhalation of a gas mixture containing 45% oxygen, 53.5% air and 1.5% isoflurane (Forene; Abbot BV, Hoofddorp, The Netherlands). A 3 cm skin incision was made parallel to the femur shaft over the trochanter major of the right femur. After splitting the fascia

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
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<tbody>
<tr>
<td>Dhvar-5</td>
<td>12.5</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>≥256</td>
</tr>
<tr>
<td>Penicillin</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>256</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>≥256</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>256</td>
</tr>
</tbody>
</table>

Values were determined by the broth dilution method in BHI for Dhvar-5 and the disc diffusion method for the other antibiotics.37
and retracting the femoral biceps, the trochanter was exposed and the bone was notched to mark the drill site. The cortex was penetrated with a small drill (2.0 mm diameter), which was gradually increased to 3.5 mm diameter. The femoral canal was reamed with small stainless-steel brushes (Abrasive Center, Maastricht, The Netherlands) increasing in size up to 4.0 mm diameter. Subsequently the canal was flushed with 50 mL of 0.9% NaCl solution to remove any bony debris created by the reaming procedure. A small suction cuvette was inserted into the medullary canal to remove as much blood and NaCl solution as possible. In quick succession, the bacterial inoculation (0.1 mL) was injected and the beads inserted (Figure 1). The fascia and skin were closed with Vicryl 4/0 as soon as possible to prevent leakage of the inoculum. Post-operative pain relief was provided by injection of 0.15 mg of buprenorphine subcutaneously, which was repeated if necessary.

Autopsy and sample acquisition

After 7 days, the animals were euthanized, first by sedation with a subcutaneous injection of 2.5 mg/kg xylazine and 62.5 mg/kg ketamine, and then by killing with an intravenous injection of 75 mg/kg pentobarbital (Nembutal; CEVA Sante Animale, Naaldwijk, The Netherlands). Blood samples were drawn for WBC and ESR determination before administration of the pentobarbital. The right and left (control) femur were excised under strict aseptic conditions. After excision, radiographs of all femora were taken to document possible signs of osteomyelitis. Then a transverse saw cut was made in the middle of the femur, care being taken not to damage the bead chain inside the femoral canal. The bead chain was then gently removed from the femoral cavity. Subsequently, transverse sections were saved from all proximal femora, for histological and bacteriological analysis (Figure 1).

Bacterial cultures

A transverse section of approximately 1 g from the proximal half of the femur was weighed and then homogenized in 50 mL of PBS at 15,000 rpm for 2 min (Sorval Omnimix; Dupont Instruments, Newton, CT, USA). A serial dilution was plated on blood agar and incubated for 24 h at 37°C. The bacterial load was expressed as cfu per gram of bone tissue. The detection limit of this method was approximately $2.5 \times 10^3$ cfu/g of bone depending on the mass of the specific homogenized bone section. For statistical analysis, the samples with no growth of bacteria in the quantitative assay were considered to have the detection limit value ($\sim 2.5 \times 10^3$ cfu/g of bone) of bacterial load. Additionally, in order to assess the possible presence of MRSA below the detection limit of the quantitative method, a 100 µL sample of bone homogenate was cultured in BHI broth for at least 48 h at 37°C.

Results

General

All animals tolerated and recovered well from surgery. However, one animal from the 12 mg Dhvar-5 group and two from the 24 mg Dhvar-5 group sustained a peroperative trochanter fracture and were thus excluded from the study. Also, one control rabbit was excluded due to a superficial infection. In total, 32 rabbits were included for statistical analysis.

There were no significant differences in weight or in temperature between the different groups (Figure 2a and b). Within groups, the 12 mg Dhvar-5 group lost 3.8% of its body weight ($P = 0.005$), and the control group showed a minor increase in temperature of 0.5°C ($P = 0.05$); no other significant differences were found.

Also, there were no differences in the WBC values between groups preoperatively and at day 7 (Figure 2c). Within groups, the WBC increased significantly from preoperative to day 7 values in both the control group ($P = 0.05$) and the 12 mg Dhvar-5 group ($P = 0.01$). There were no differences in ESR between groups except for the 12 mg Dhvar-5 group (Figure 2d), which had a significant increase and was higher compared with the other groups ($P = 0.04$). As expected, after 7 days follow-up, the radiographs did not show signs of infection (Figure 1).

Microbiology

Quantitative data of the cultures from the femora are shown in Figure 3. In the sham-treated control group a mean (±S.E.M.) of $1.9 \times 10^6$ (±0.8 $\times 10^5$) cfu/g of bone ($n = 7$) were cultured and in the 12 mg ($n = 7$) and 24 mg ($n = 7$) Dhvar-5 groups, $0.78 \times 10^6$
(±0.2 \times 10^6) and 0.51 \times 10^6 (±0.1 \times 10^6) cfu/g of bone were cultured, respectively. The difference between the control group and the 12 mg Dhvar-5 group was significant (P = 0.04); between the 12 mg Dhvar-5 group and the control group the difference almost reached significance (P = 0.07). Furthermore, in the 10 and 24 mg gentamicin groups means of 0.55 \times 10^6 (±0.4 \times 10^6) cfu/g of bone and 0.09 \times 10^6 (±0.08 \times 10^6) cfu/g of bone were cultured, respectively. The difference between the control group and the 10 mg gentamicin group almost reached significance (P = 0.06), while the 24 mg gentamicin group had a significantly lower colony count compared with the control group (P = 0.02) and compared with the 24 mg Dhvar-5 group (P = 0.04). All left femora (not operated control) did not show any growth of bacteria.

Bacteria were retrieved from all control animals and from all Dhvar-5-treated animals (Table 2). Successful prevention of osteomyelitis indicated by no growth of bacteria in BHI cultures after 48 h was achieved in 3/7 and 3/5 animals of the 10 and 24 mg gentamicin groups, respectively.

![Figure 2](image1.png)

**Figure 2.** Body weight (a) and temperature (b) were measured preoperatively (white bars) and at 7 days follow-up (black bars) (values are mean ± S.D.). The 12 mg Dhvar-5 group suffered mild weight loss (P = 0.005) and the control group had a slight increase in temperature (P = 0.05). There were no other significant differences between the groups in terms of weight and temperature. The WBC (c) increased significantly in the 12 mg Dhvar-5 group (P = 0.01) and the control group (P = 0.05). Furthermore, the ESR (d) increased significantly from the preoperative value to the day 7 value in the 12 mg Dhvar-5 group and was significantly higher compared with the other groups (P = 0.04). No preoperative differences were seen between groups.

![Figure 3](image2.png)

**Figure 3.** Quantitative microbiological analysis. Average counts of cfu per gram of bone tissue (+S.E.M.) at day 7 are plotted. The 24 mg Dhvar-5 and 24 mg gentamicin groups significantly reduced the bacterial load compared with the control group (P = 0.04, bP = 0.02). Also, the 24 mg gentamicin group reduced the cfu count more than the 24 mg Dhvar-5 group (P = 0.04). Both the 12 mg Dhvar-5 and the 10 mg gentamicin groups showed a trend towards reducing the bacterial load; P = 0.07 and P = 0.06, respectively.

<table>
<thead>
<tr>
<th>Bead chain</th>
<th>MRSA ((\times 10^6))</th>
<th>periosteal reaction</th>
<th>bone necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7/7</td>
<td>7/7</td>
<td>6/7</td>
</tr>
<tr>
<td>12 mg Dhvar-5</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>24 mg Dhvar-5</td>
<td>7/7</td>
<td>6/7</td>
<td>6/7</td>
</tr>
<tr>
<td>10 mg Gentamicin</td>
<td>4/7</td>
<td>5/7</td>
<td>7/7</td>
</tr>
<tr>
<td>24 mg Gentamicin</td>
<td>2/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
</tbody>
</table>

Table 2. Qualitative microbiological data are shown as the number of animals per group from which bacteria were cultured in BHI after 48 h at 37°C. Furthermore, the number of animals with early histopathological signs of osteomyelitis are described per group.
Table 2 also presents the histopathological results. In control animals, minor periosteal reaction (Figure 4a and b) was seen in all animals (7/7) and femoral necrosis in 6/7 animals. Similar results were seen in the 12 mg and 24 mg Dhvar-5 groups with a minor periosteal reaction in 6/6 and 6/7 animals, respectively. Bone necrosis was found in all but one of the 24 mg Dhvar-5 treated group. One animal of the 24 mg Dhvar-5 group had no signs of osteomyelitis. In the 10 mg gentamicin group, 5/7 animals showed a periosteal reaction and 7/7 showed bone necrosis. In the 24 mg gentamicin group, 3/5 animals showed early signs of osteomyelitis. The histopathological scores revealed no significant differences between the groups (data not shown).

Discussion
In this study, we used a prophylactic model which was modified from the model described by Nijhof et al. Instead of self-setting PMMA bone cement we used custom-made PMMA beads of the same dimensions (3 x 5 mm) as the commercially available Septopal mini-beads (Biomet Merck). These mini-beads were then inserted into the inoculated femoral canal (Figure 1). The MRSA strain was a clinical isolate from a patient with osteomyelitis and therefore should have had sufficient virulence to consistently generate osteomyelitis in vivo. To further ensure a reproducible infection model with 100% infection in sham-treated control animals, the number of cfu injected was increased to 10^7 cfu, as opposed to the 10^6 cfu described by Nijhof et al. These measures resulted in a 100% infection rate in a the sham-treated control group.

Figure 4. (a) Survey micrograph of a transverse section from a control femur (fuchsin and Methylene Blue, magnification 25×). The arrowheads indicate remnants of the blood clot which still imbeds the beads after 7 days. An early periosteal reaction can be seen (arrows). (b) Detail of the periosteal reaction (magnification 200×); this phenomenon was also seen in other groups (Table 2). (c) High magnification (200×) of a control femur; necrotic bone can be identified by empty lacunae (arrowheads). (d) Gentamicin-treated femur without periosteal reaction (magnification 200×); cultures of this femur also showed no bacterial growth. A colour version of this figure is available at JAC Online.
Efficacy of Dhvar-5 mini-beads in vivo

An advantage of this model is the minor systemic signs of infection (Figure 2a and b). Despite the relatively large inoculum (10^9 cfu), none of the operated animals developed sepsis. This was also reflected in the systemic infection parameters, which showed only minor changes in all groups (Figure 2c and d). Another advantage of this study is the good and reproducible distribution of PMMA beads throughout the femoral canal (Figure 1), the number and proximity of antibiotic beads is well known to influence antibiotic tissue levels in the bone. Local treatment of osteomyelitis consists of debriding necrotic bone and inserting as much as possible antibiotic beads into the medullary canal, adding to the clinical relevance of this model.1

The data presented show that 24 mg of Dhvar-5 incorporated in custom-made PMMA mini-beads can inhibit MRSA proliferation in vivo, significantly reducing bacterial load compared with controls (P = 0.04) (Figure 3). The 12 mg group also demonstrated a trend (P = 0.07) towards reduction in bacterial load per gram of bone. The data therefore suggest a dose–response effect of Dhvar-5 in vivo (Figure 3). Still, even a high dose of Dhvar-5 was not able to eradicate all bacteria and sterilize the femur. The 24 mg gentamicin group on the other hand showed inhibition of MRSA proliferation (P = 0.02) and sterilized three out of five femora (Table 2 and Figure 3). The low-dose gentamicin (10 mg) group showed a trend (P = 0.06) towards reducing the number of cfu per gram of bone and sterilized three out of seven femora.

The MRSA strain was highly resistant to several antibiotics including gentamicin (MIC = 256 mg/L, Table 1). Despite this marked resistance, the high-dose gentamicin beads did prevent infection in three of five rabbits. Several studies have shown that the local application of gentamicin in PMMA beads can result in local bone tissue levels around this high MIC value,2,22 thus explaining the ability of the gentamicin beads to sterilize some of the femurs in this study.

There are numerous reports indicating an increase in antimicrobial resistance, in particular of S. aureus and coagulase-negative staphylococci (CoNS).3,4,23–25 In addition to the high prevalence of MRSA, the first strains of vancomycin-resistant S. aureus (VRSA) are now being reported.5 Moreover, the development of antimicrobial resistance is increased in biofilms.5 The formation of biofilms renders bacteria very resistant to conventional antibiotics and can even result in bacteria colonizing gentamicin-containing PMMA beads.5 These developments have increased concern over the future applicability of conventional antibiotics, and spurred the development of novel antimicrobial agents.7,8,27–34

Over the past decade, numerous naturally occurring AMPs have been identified and their pivotal role in the mechanism of our innate immune system has been discovered.13 Several research groups have specifically described options for the development of AMPs as new antimicrobial agents.1,8,27–34 The rBPI12, antimicrobial peptide has reached Phase III clinical trials and has shown promising clinical results: improving the outcome of meningococcal sepsis in children.28

Based on our in vitro release study, we first chose to incorporate 12 and 24 mg of Dhvar-5 in the PMMA beads,15 as this should have resulted in a continuous release of a sufficient quantity of Dhvar-5 to kill bacteria at the inoculated site for the duration of the experiment. After 7 days of release in vitro, 65% of Dhvar-5 is released from the 24 mg chain as opposed to 37% of gentamicin from the 24 mg gentamicin beads (ref. 15; and

C. Faber, R. J. W. Hoogendoorn, D. M. Lyaruu, H. P. Stallmann, J. van Marle, A. V. Nieuw Amerongen, T. H. Smit & P. I. J. M. Wuisman, unpublished results). The high dose of Dhvar-5 resulted in a significantly reduced bacterial load per gram of bone (Figure 3). However, as there was no method available to measure in vivo concentrations of Dhvar-5, we could not determine the systemic or local concentrations of Dhvar-5. It is therefore difficult to relate the very high release characteristics in vitro of Dhvar-5 to the results obtained in vivo. There are no data available on the in vivo half-life of Dhvar-5. Other AMPs are known to have a much shorter half-life than gentamicin.20 It is therefore possible that Dhvar-5 is cleared more rapidly than gentamicin. Also, in vitro studies have shown that Dhvar-5 can be degraded by proteolytic enzymes such as trypsin.25 This could possibly reduce local tissue levels of Dhvar-5 in vivo. Furthermore, some AMPs have been reported to bind to serum proteins, resulting in reduced antimicrobial activity.36 In this study, a combination of these factors could have resulted in lower local free and/or active tissue concentrations of Dhvar-5, possibly compromising the in vivo efficacy, thus explaining the relatively better performance of the gentamicin beads compared with the Dhvar-5 beads, despite the marked gentamicin resistance of the MRSA strain.

This study demonstrates the in vivo antimicrobial activity of Dhvar-5 against MRSA. However, the gentamicin beads demonstrated the best efficacy in preventing MRSA osteomyelitis in this study. Future development of Dhvar-5 and other AMPs as novel antimicrobial agents is largely dependent on the ability to control aspects such as the pharmacodynamics, inhibition of enzymic proteolysis and prevention of possible inactivation by binding to proteins. Numerous opportunities can be found in changing amino acid sequences whilst retaining or even improving the antimicrobial activity. This research provides a basis for the further development of AMPs as an alternative for conventional antibiotic agents for the treatment of drug-resistant pathogens.

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Supplementary data

Supplementary data accompanying this paper are available at JAC Online.

References


