Evaluation of comparative soft tissue response to bone void fillers with antibiotics in a rabbit intramuscular model

Rema A Oliver, Vedran Lovric, Chris Christou and William R Walsh

Abstract
Management of osseous and soft tissue dead space can be a significant challenge in the clinical setting. Calcium sulphate and calcium phosphate-based biomaterials are increasingly being used as alternatives to PMMA for local release of antibiotics, in particular to fill dead space following surgical debridement. This study aims to observe the in-vivo absorption characteristics and tissue response of three commercially available calcium sulphate-based materials combined with gentamicin in an established soft tissue rabbit model. The implant materials (1cc) were placed into four intramuscular sites in 18 New Zealand White rabbits (n = 6). In-life blood samples and radiographs were taken from each animal following implantation. Animals were sacrificed at 0, 1, 7, 21, 42 and 63 days post-operatively (n = 3) and implant sites analysed by micro-computed tomography and histology. Radiographically and histologically, recrystallized calcium sulphate (RCS) absorbed the fastest with complete absorption by day 21. Calcium sulphate/HA composite (CSHA) and Calcium sulphate/calcium carbonate (CSCC) absorbed slower and were detectable at day 63. Residual bead analysis revealed the presence of detectable gentamicin at 24 h and 7 days for CSHA and RCS but none in CSCC. Systemic levels of gentamicin were only detected between 1 h and 24 h. Serological inflammatory cytokine expression for IL-6, TNF-α and IL-1β indicated no unusual inflammatory response to the implanted materials. Calcium sulphate materials loaded with gentamicin are effective in resolving a surgically created dead space without eliciting any adverse host response.

Keywords
Bone void filler, gentamicin, soft tissue reaction, dead space management

Introduction
Calcium sulphate and calcium phosphate-based biomaterials are increasingly being used as alternatives to polymethylmethacrylate (PMMA) for local release of antibiotics in particular to fill dead space following surgical debridement in the management of infection. These materials are biocompatible and are absorbed by the body negating the need for removal while resorption rates can vary based on chemistry. Due to the low temperatures achieved during setting, they can be mixed with heat sensitive antibiotics. Clinical reports of composite materials incorporating calcium phosphates such as hydroxyapatite (HA) with calcium sulphate were found to demonstrate soft tissue healing in the treatment of the infected diabetic foot with no foreign body or immune host response. Published absorption rates of these materials are inconsistent with both complete absorption being reported as in the data above and partial dissolution and bony incorporation of the HA particles in other literature.
PMMA, however, is not absorbed in the body and therefore is required to be removed in a second procedure to decrease the possibility of the material becoming a nidus for infection. Local implantation of gentamicin impregnated PMMA beads can provide high local levels of antibiotic, many times the minimum inhibitory concentration (MIC) while reducing the risk of systemic toxicity. Gentamicin is stable when exposed to the high temperatures generated during the polymerisation reaction as the PMMA sets hard, therefore it is commonly used in the local treatment of osteomyelitis and soft tissue infections.

The purpose of this study was to observe the in-vivo absorption characteristics and tissue response of three commercially available calcium sulphate-based materials combined with gentamicin based on a previously described novel soft tissue animal model where the materials were implanted in four non-adjacent intramuscular sites in adult rabbits.

**Methods**

**Preparation of implant materials**

Three commercially available materials were used for this study as shown in Table 1.

The hemihydrate powder from 10cc kits for the recrystallized calcium sulphate (RCS) beads was mixed with 6 ml (240 mg) gentamicin solution (40 mg/ml, Hospira, UK) and was prepared under sterile conditions. The mix was thoroughly blended for 30 s to form a smooth paste which was then pressed into 6 mm diameter, 4.8 mm length, hemispherical cavities in a flexible mould. The beads were left undisturbed and allowed to set. The level of gentamicin combined with the RCS corresponded to the clinical ratio combined with RSC reported in literature.

The 10cc kits of CSHA were mixed according to the manufacturer’s Instructions For Use (IFU) with the gentamicin included in the pack at a concentration of 175 mg per 10cc. The mixed paste was then pressed into 6 mm diameter, 4.8 mm length, hemispherical cavities and allowed to set as described above. When all the beads had set hard, they were removed by flexing the mould. The level of gentamicin combined with CSHA was supplied co-packaged with the product, for combination with the product according to the manufacturer’s instructions.

The CSCC beads were supplied as pre-formed white to light grey beads of biconvex rounded cylindrical shape. Each bead weighed 250 mg containing gentamicin at a concentration of 1%, equivalent to 2.5 mg gentamicin per bead. The CSCC beads were supplied preloaded with gentamicin.

**Surgery**

Following approval of the Animal Care and Ethics Committee of the University of New South Wales (ACEC#:15/85A), implant materials (1cc per side, five beads of material) were placed into intramuscular sites in 18 female New Zealand white rabbits (average weight 3.5 kg, aged 7–9 months old), 6 rabbits per material. For all 18 animals in the study, four implant sites were used per animal, two sites each side of the spine, in non-adjacent intramuscular sites (longissimus muscles) above the spine at the levels L1–L2, L2–L3, L3–L4 and L4–L5. Under gaseous anaesthesia of isoflurane and oxygen, the intermuscular plane between the multifidus and longissimus muscles was retracted to create a 1 cm × 2 cm void. Each void was filled with 1cc of sterile beads. The beads were counted at the time of surgery and were allocated in a sterile fashion into sterile syringes with the tip removed to facilitate implantation.

Each of the facial incisions was closed with an individual single strand non-absorbable suture. Closure was achieved with equidistant adjacent stitches at approximately 3 mm intervals. The skin of the incision was closed with an individual single strand absorbable suture. Post-operative radiographs in the posteroanterior and lateral planes were taken immediately following surgery using a mobile X-ray machine (Poskom Co., Ltd, Korea) and digital cassettes (AGFA, Sydney, Australia). Radiographs were used to visualise the appearance of the beads in the soft tissue post-
operatively. These images were used as a baseline for examination at later time points.

**Peripheral blood analysis and blood serum gentamicin levels**

Peripheral blood was taken preoperatively and 1, 6, 12 and 24 h following surgery and prior to sacrifice at each time point for standard blood panel haematology/biochemistry (IDEXX Laboratories, Sydney, Australia) and serum cytokine levels for IL-1\(\beta\), IL-6 and TNF-\(\alpha\) and to determine systemic gentamicin levels.

Peripheral blood (approx. 3 ml) was taken from the saphenous vein and collected into a Vacutainer Plain Clot 4 ml tube and allowed to clot at room temperature for 30 min before centrifugation for 10 min at 2000 \(\times\) g. The serum was carefully removed and stored at \(-80^\circ\)C until needed.

Serum IL-1\(\beta\) (E04I0010), IL-6 (CSB-E06903Rb) and TNF-\(\alpha\) (CSB-E06998Rb) levels were analysed by enzyme-linked immunosorbent assay (ELISA) testing using ELISA kits (Cusabio Biotech, Beijing, China) according to the manufacturer’s instructions. Each sample was measured in duplicate and standards were also run on each 96-well plate.

**Blood serum gentamicin levels – Method validation in-vitro**

Rabbit serum was harvested from other studies at the time of sacrifice to perform a dose response curve for rabbit serum with a known concentration of gentamicin. Concentrations were determined based on the assay range and also included concentrations lower and higher than the detectable assay range. The study was completed with a standardised assay. This was performed in duplicate. The antibiotic used was Gentam 100 (100 mg/ml gentamicin as gentamicin sulphate, Troy Laboratories, NSW, Australia).

The assay was based on the kinetic interaction of microparticles in a solution (KIMS) where the gentamicin antibody is covalently coupled to microparticles and the drug derivative is linked to a macromolecule. The kinetic interaction of microparticles in solutions is induced by binding of drug-conjugate to the antibody on the microparticles and is inhibited by the presence of gentamicin in the sample. A competitive reaction takes place between the drug conjugate and gentamicin in the serum sample for binding to the gentamicin antibody on the microparticles. The resulting kinetic interaction of microparticles is indirectly proportional to the amount of drug present in the sample. The assay range was 0.4–10 \(\mu\)g/ml. Several data points were also run outside of the range to challenge the assay. The serum samples collected post-implantation were analysed using this assay.

**Euthanasia and necropsy**

Time points for sacrifice were time 0, and days 1, 7, 21, 42 and 63. Each time point had three animals with four implantation sites per animal. Time points were chosen to examine the in-vivo release kinetics of gentamicin in local tissues as well as serum levels.

Implant sites were reviewed for general integrity of the skin incision along with the macroscopic reaction of the underlying subcutaneous tissues as normal or abnormal. The abnormal was further assessed as evidence of infection or macroscopic signs of inflammation/foreign body reaction. At the time of harvest, all organs were examined and any abnormalities noted. A portion of the distant organs was processed for routine paraffin histology and evaluated in a blinded fashion for any abnormalities.

**Radiography and micro-computed tomography**

Post-operative radiographs in the posteroanterior plane were taken using a mobile X-ray machine and digital cassettes. These images were used to determine radiographic absorption by comparison to radiographs obtained immediately after implantation.

Micro-computed tomography (microCT) was performed for all animals using an Inveon in-vivo microcomputer tomography scanner (Siemens Medical, PA, USA) in order to obtain high resolution images of the implant absorption. The surgical sites were scanned and the raw images reconstructed resulting in effective pixel size of 53.12 \(\mu\)m. Images were examined in the axial, sagittal and coronal planes and 3D models were created using Siemens image analysis software (Inveon Research Workplace 3.0, Siemens Medical, PA, USA).

**Gentamicin levels in residual materials and adjacent muscle tissues**

The surgical sites were carefully dissected and examined for the presence of any residual beads. A portion of any residual beads present at the surgical sites was harvested. This material was allowed to air dry and placed in a desiccator for 24 h. Following this, they were then morselized using a mortar and pestle. 0.1 g of the powder was immersed in 1 ml of serum for 24 h. Gentamicin levels in the samples per gram of material were determined using the assay described above.

A muscle sample (1 cm \(\times\) 1 cm) at the implantation site was harvested and then minced. The local concentration of gentamicin in the muscle sample was
measured using the KIMS standard antibody assay already described.

**Histology**

Harvested implant sites were immediately fixed in phosphate buffered formalin for a minimum of 48 h followed by decalcification in 10% formic acid – phosphate buffered formalin at room temperature. The decalcified samples were placed into embedding blocks for paraffin processing. Paraffin blocks were sectioned using a microtome (Leica, Germany) to 5 microns and placed onto slides for routine haematoxylin and eosin (H&E) staining.

Stained sections were reviewed and photographed using an Olympus Microscope (Olympus, Japan) and Olympus DP72 Camera. In-vivo response and biocompatibility to the materials was assessed at the implant site/host tissue boundary in a blinded manner.

**Results**

Surgery was completed without any adverse events. All animals recovered following surgery.

On harvest, macroscopic observations revealed the skin, subcutaneous tissue and organs to all be normal.

**Blood serum gentamicin levels**

Results of measured gentamicin levels for each rabbit at each allocated blood sampling time point for each material are shown in Figure 1. Systemic levels of gentamicin were only detected at the time points between 1 h and 24 h. The maximum detected level was 9 μg/ml at the 6 h time point.

**Blood serum inflammatory cytokine level (ELISA) analysis**

The levels of systemic cytokines were quantified preoperatively (baseline) n = 6 then postoperatively at 1 h, 6 h and 12 h (n = 5). Further levels were taken prior to sacrifice for each animal at 1 day, 7 days, 21 days, 42 days and 63 days (n = 1). The levels were determined in comparison to standard curves for IL-6, IL-1β and TNF-α.

Inflammatory marker IL-1β was detected in all animals at each time point including those in the RCS group where no further material remained. IL-6 was only detected up to and including the 12 h time point. The TNF-α results were inconclusive and not detected in all animals in all groups. The data presented in Figures 2 to 4 represented the mean values where the cytokines were detected. In days 1 to 63 only single samples were reported.

**Tissue sample harvesting and gentamicin levels**

Analysis of the residual beads revealed the presence of detectable gentamicin at 24 h and 7 days for CSHA and RCS. No gentamicin was detected in the CSCC samples at seven days. Gentamicin was not detectable in the residual beads in the CSHA and CSCC groups at

![Figure 1. Systemic gentamicin levels. Mean values shown. n = 3. Error bars show standard deviation.](image-url)
day 21 or day 42 despite material remaining. As there was no presence of RCS beyond day 21 this was not evaluated (Table 2). No gentamicin was detected in the muscle samples.

**Radiography**

RCS demonstrated the most rapid absorption profile with changes observed by day 7 and the material radiographically almost completely absorbed by day 21.

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**Figure 2.** Serological cytokine expression for IL-1β. IL-1β levels were detected at each time point in all groups. The detection range of the assay was 78–5000 pg/ml. Error bars are only shown up to the 12 h time point as only single samples were obtained for the later time points.

**Figure 3.** Serological cytokine expression for IL-6. IL-6 levels only detected up to the 12 h time point. The detection range of the assay was 15.6–1000 pg/ml.
CSHA and CSCC absorbed slower based on radiographs and were detectable in all four implantation sites at day 63. The materials also did not appear to migrate based on the serial radiographs (Figures 5 to 7).

**Micro-computed tomography**

Imaging by microCT provided a clear assessment of bead absorption and confirmed the radiographic findings. Representative microCT images are shown in Figures 8 to 10. There was a clear progression of absorption over time with all implanted beads. This absorption appeared to be occurring from the outside in. In some cases at the latter time points, a ‘halo’ was observed surrounding the remaining beads, extending out into the surrounding soft tissue. There was also a clear difference in the rate of absorption between each type of implanted bead.

MicroCT indicated that RCS had the most rapid absorption profile with changes observed by day 7 and the material almost fully absorbed by day 21. CSHA and CSCC absorbed slower and were detectable in all four implantation sites at day 63.

**Histology**

The histological reaction demonstrated a subtle inflammatory response for all materials at the host interface versus time that included some lymphocytes and the occasional multinucleated cell (Figure 11). The overall intensity of the reaction was minor and resolved with time for all materials.

The histology beyond day 21 for RCS was unremarkable and by day 63 was normal in the implantation sites. The CSHA and CSCC samples presented slower absorption profiles and a longer presence of material in-vivo. This was paralleled with the presence of a few lymphocytes and multinucleated cells while the material remained. Complete absorption of these two materials by day 63 was not achieved (Figure 12).

**Discussion**

The animal model used in this study provided a robust means to evaluate intramuscular implantation of calcium sulphate materials combined with antibiotics in a pre-clinical setting. All animals recovered well following surgery with no in-life adverse events. Inspection of the wounds at the time of harvest revealed no adverse effects for the skin incision. All implanted beads could be clearly visualised on the radiographs and microCT images. There was a clear progression of absorption over time with all beads with no radiographic signs of migration. This absorption appeared to be occurring from the outside in. In some cases at the latter time points...

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*Figure 4.* Serological cytokine expression for TNF-α. The detection range of the assay was 7–1000 pg/ml. Error bars are only shown up to the 12 h time point as only single samples were obtained for the later time points.

| Post-op | 24 h | 7 Days | 21 Days | 42 Days |
|---------|------|--------|---------|---------|
| RCS     | 10.66| 4.215  | 4.405   | N/A     | N/A     |
| CSHA    | 8.29 | 6.37   | 4.39    | 0       | 0       |
| CSCC    | 1.115| 0.65   | 0       | 0       | 0       |

Two samples per group.
points, a ‘halo’ was observed surrounding the remaining beads. It is suspected that this is a temporary carbonated apatite precipitation due to the release of high levels of calcium into the surrounding tissue which combine with ions in situ and precipitate on to the surface of the residual beads as has been observed in vitro.\textsuperscript{17,18} There was a clear difference in the rate of absorption between each type of implanted bead.

Figure 5. Representative RCS Radiographs demonstrating bead absorption. RCS had almost completely absorbed by day 21 and resorbed via surface absorption.

Figure 6. Representative CSHA Radiographs demonstrating bead absorption. Residual material was still present at day 63 and resorbed via surface absorption.

Figure 7. Representative CSCC Radiographs demonstrating bead absorption. Residual beads were still present at day 63 and resorbed via surface absorption.
Figure 8. Representative RCS microCT images demonstrating bead absorption. The material was almost completely absorbed by day 21.

Figure 9. Representative CSHA microCT images demonstrating bead absorption. Residual material was still present at day 63.

Figure 10. Representative CSCC microCT images demonstrating bead absorption. Residual material was still present at day 63.
RCS demonstrated the most rapid absorption profile with changes observed by day 7 and the material radio-graphically almost completely absorbed by day 21. CSHA and CSCC absorbed slower and were detectable in all four implantation sites at day 63. Residual material was also apparent on explantation. Analysis of the residual beads revealed the presence of detectable gentamicin at 24 h and 7 days for CSHA and RCS. No gentamicin was detected in the residual CSCC samples at seven days. Furthermore, gentamicin was not detectable in the residual beads in the CSHA and CSCC groups at days 21 or 42, despite material remaining.

As seen with PMMA beads, once levels of released antibiotics are below MIC, any residual material may present a nidus for infection. In this case any gentamicin-resistant bacteria could colonise unabsorbed materials.

The clinical use of calcium sulphate and calcium phosphate-based materials for the local release of antibiotics has been reported in indications where infection is present, ranging from diabetic foot infections to orthopaedic indication such as periprosthetic joint infections and trauma. PMMA has been widely reported, with clinicians frequently trying to achieve optimal antibiotic loading without compromising mechanical properties of the cement, particularly when the cement is being used as a spacer in two stage revision surgery or for prosthesis fixation. Unlike PMMA where the majority of antibiotic remains locked within the polymer, the combination of absorbable materials with antibiotics has the potential advantage of being able to release the entire antibiotic dose with which it is combined.

In-vitro studies measuring antibiotic release from absorbable materials have reported antibiotic elution being maintained at concentrations more than 500 μg/ml at 42 days, but there is a large variation in the in-vitro experimental methods used to determine antibiotic elution from absorbable materials. Methodologies vary with respect to a number of parameters, including the volume removed for analysis and the eluent sampling intervals, and the quantity of material tested. In addition the nature by which the sample is presented to the solution can have an effect, with elution from a single small bead will typically have lower antibiotic concentrations with elution for a shorter duration than if a larger cast cylinder of material is used.

**Figure 11.** Haematoxylin and eosin (H&E) staining. Histology at day 1 for the RCS and the CSHA groups revealed the start of an initial inflammatory response at the margin with the host muscle. The initial cellular population included lymphocytes. Histology at day 1 for CSCC showed an overall lack of any initial inflammatory reaction. The materials are labelled with a star.
The clinical evaluation of antibiotic release from hydroxyapatite/calcium sulphate composite has reported levels of gentamicin were still present in urine 60 days post-surgery. Another study has evaluated the local and systemic antibiotic levels in patients implanted with vancomycin-loaded calcium sulphate, reporting local concentrations were approximately ten times higher than with polymethylmethacrylate (PMMA) as a carrier, whilst serum levels typically remained less than 10 mg/l in the first days following implantation, decreasing rapidly.

Upon implantation of a biomaterial into a surgical site, various reactions take place including foreign body response and inflammatory reactions. No adverse reactions to the implanted materials were noted. Histologically, all materials were well tolerated versus time. The inflammatory response included some lymphocytes and multinucleated cells that resolved with time for all materials.

The use of calcium sulphate in soft tissue sites suggests good tissue compatibility and complete absorption with minimal complications. Kallala et al. reported on the use of calcium sulphate beads in 755 cases of revision total hip and total knee arthroplasty with 4.2% drainage and 1.7% heterotopic ossification.

Swords et al. details the use of calcium sulphate as an intracorporal cast for the treatment of infected penile implants acting as a filler, preventing fibrosis and loss of space with full absorption in four to six weeks and uneventful postoperative follow-up. Sherif et al. carried out a retrospective analysis on the use of antibiotic-loaded calcium sulphate beads to salvage infected breast implants with positive outcomes and Kenna et al. presented data using absorbable antibiotic beads for prophylaxis in immediate breast reconstruction in 68 patients, reducing the risk of periprosthetic implant infection with no complications. Healy et al. reported on the direct placement of antibiotic-loaded calcium sulphate beads in the management of prosthetic vascular graft infections with dissolution in approximately six weeks.

Raina et al. observed bone formation in the overlaying muscle covering surgically created bone defects when a calcium sulphate/HA composite material was used, implying that the combination of inductive proteins released from a defect in apposition to an osteoconductive material can enhance the process of ectopic ossification. Pre-clinical work by Wang et al. investigated the reaction to the implantation of a calcium sulphate/HA composite material with and without the
addition of autologous bone marrow in rat muscle, evaluating the absorption and soft tissue reaction. Signs of an inflammatory reaction were noted and material remained at 12 weeks and no muscle necrosis was observed.

In contrast to these data, the release of HA particles by bone substitutes or as a coating on implants has previously been shown to induce an inflammatory response. The cellular response to three types of calcium phosphate was investigated by van der Meulen et al. and found that all materials produced a short mild inflammatory reaction. Mestres et al. reported that hydroxyapatite substances can influence the growth and proliferation of macrophage-like cells. Recorded cases of implanted materials leaking from filled cavities causing tissue reactions have been reported and recorded by the FDA on the MAUDE database.

RCS produced a reliable and reproducible in vivo resorption based on radiographs and microCT and was not detected on day 21. While some heterotopic ossification has been reported with calcium sulphate with vancomycin and tobramycin, this was not noted in the present study. The histology results for RCS versus time revealed the material to be very well tolerated in vivo in this model. The local inflammatory cells present at the interface with the muscle in this model, while the material was absorbing, resolved with time and normal tissue was present in the implantation sites from 42 days.

Differences were noted for the in vivo absorption of the three materials. The CSHA and CSCC beads both demonstrated material remaining at days 42 and 63 with evidence of persistent lymphocytic activity. For the CSHA beads, the remaining material was suspected to be the HA component of the material. The remaining material in the CSCC group warrants further investigation, as does, the persistent “halo” of extending into the surrounding soft tissue at the final follow-up for both the CSHA and CSCC. For both these groups, the presence of gentamicin was not detected in the residual material.

The in vivo absorption of calcium sulphate has been investigated since the early 1960s. In the study of materials used to fill osseous defects, Bell demonstrated that plaster of Paris was absorbed twice as fast as autogenous bone and many times faster than homologous and heterogeneous bone when implanted in well vascularized gastrocnemius muscles. He reported complete absorption of plaster in approximately 33 days.

Research has also studied the subcutaneous implantation of calcium sulphate into 12 Sprague-Dawley rats to determine the rate of material degradation in vivo and the reaction of the surrounding tissues. Results indicated a localized reaction to the material as it degraded in the tissue, yet there was proliferation of granulation tissue which matured into a dense scar with little surrounding tissue reaction. The majority of material was absorbed within eight weeks.

Conclusions from this study were that when implanted into subcutaneous sites, calcium sulphate resorbed too rapidly to be effective in inducing bone replacement. The in vivo mechanism of absorption for calcium sulphate has been investigated. Ricci observed that calcium sulphate materials were absorbed by rapid dissolution, both in vitro and in vivo, noting absorption from the outer surface inwards, at up to 1 mm per week. The absorption from outside in is observed in our study with RCS which supports Ricci’s observations.

Based on the data presented here and reported clinical use, RCS when implanted into soft tissue is completely absorbed and inflicts a minor inflammatory response which resolves as the material is absorbed. CSHA and CSCC also demonstrate a minor inflammatory response but the presence of residual material demonstrates a slower absorption profile and the complete absorption of both these materials was not achieved at the time points evaluated here.

This study had a number of limitations. No sham control animals were employed in the model to determine the surgical site response of an unfilled intramuscular site. It was felt, that this would have provided limited information, as the intramuscular site would have closed with a dead space formation if no implant material was placed. A control group that included PMMA mixed with gentamicin was also not employed. In this study we were primarily focusing on calcium sulphate-based biomaterials. Lastly, the model as described is not an infection model. Hence it was not possible to determine the in vivo efficacy of the antibiotic in the biomaterials on protecting the material from bacterial colonisation.

**Conclusion**

All materials were well tolerated, with no adverse host responses observed. All materials released gentamicin on implantation and were effective in resolving surgical dead space in this animal model. RCS demonstrated the most rapid absorption profile, showing almost complete absorption by day 21. CSHA and CSCC absorbed slower and were detectable in all four implantation sites at day 63. Analysis of the residual beads revealed the presence of detectable gentamicin at 24 h and seven days for CSHA and RCS. No gentamicin was detected in the residual CSCC samples at seven days. No gentamicin was detectable in residual beads of CSHA and CSCC groups at days 21 or 42. If implanted into an infected surgical site, unab sorbed
materials without residual antibiotic, or containing antibiotic at subtherapeutic levels, are at risk of colonisation by any gentamicin-resistant bacteria.

Declaration of Conflicting Interests

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ORCID iD

Rema A Oliver http://orcid.org/0000-0002-2381-7326

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