Antimicrobial Efficacy of Gentamicin-Loaded Acrylic Bone Cements with Fusidic Acid or Clindamycin Added

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ABSTRACT: The increasing gentamicin resistance among bacteria in septic joint arthroplasty has stimulated interest in adding a second antibiotic into gentamicin-loaded bone cement. A first aim of this in vitro study is to investigate whether addition of fusidic acid or clindamycin to gentamicin-loaded bone cement has an additional antimicrobial effect against a collection of 38 clinical isolates, including 16 gentamicin-resistant strains. A modified Kirby-Bauer test, involving measurement of the inhibition zone around antibiotic-loaded bone cement discs on agar plates, was used to investigate whether adding a second antibiotic has an additional antimicrobial effect. Second, a selected number of strains was used to study their survival in an interfacial gap made in the different bone cements to mimic the gap between bone and cement as existing near a prosthesis. Gentamicin-loaded bone cement had an antimicrobial activity against 58% of the 38 bacterial strains included in this study, while 68% of the strains were affected by bone cement loaded with a combination of gentamicin and clindamycin. Bone cement loaded with the combination of gentamicin and fusidic acid had antimicrobial activity against 87% of the bacterial strains. In the prosthesis-related gap model, there was a clear trend toward less bacterial survival for gentamicin-loaded bone cement after adding clindamycin or fusidic acid. Addition of clindamycin or fusidic acid into gentamicin-loaded bone cement yields an additional antimicrobial effect. The combination gentamicin and fusidic acid was effective against a higher number of clinical isolates than the combination of gentamicin with clindamycin, including gentamicin-resistant strains. © 2005 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:291–299, 2006

Keywords: antimicrobial susceptibility; acrylic bone cement; gentamicin; fusidic acid; clindamycin

INTRODUCTION

Biomaterial-related infections constitute a major threat to the current use of biomaterials. In orthopedics, use of polymethylmethacrylate (PMMA) bone cement loaded with one antibiotic to prevent biomaterial-related infections is widespread. Several studies have shown antibiotic-loaded bone cement to be beneficial in prophylaxis for primary or revision arthroplasty.[1,2] Antibiotic-loaded bone cement has been commercially available in Europe for many years. However, these bone cements were, until recently, not cleared for sale in the United States. In 2003, the FDA approved several antibiotic-loaded bone cements for the purpose of preventing further infections in two-stage joint revisions impelled by an infection of the original joint replacement prosthesis.

In general, aminoglycosides, and especially gentamicin, have turned out to be suitable

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antibiotics to incorporate in bone cement, both from a bacteriological and physicochemical point of view. Worrisome in the application of gentamicin-loaded bone cements as prophylaxis is the chance of introducing resistant strains by releasing subinhibitory antibiotic concentrations for many years. Gentamicin has remained detectable in joint fluid aspirations and tissue samples for years after using gentamicin-loaded cement for fixation of a joint prosthesis.[3,4] The introduction of gentamicin-loaded bone cements was followed within 10 years by reports on the first gentamicin-resistant staphylococci,[5–7] and nearly 50% of staphylococci responsible for prosthetic infections are now resistant to gentamicin.[8]

Due to this emerging antibiotic resistance there is a renewed interest for the addition of other antibiotics to bone cements, such as tobramycin and cefuroxime.[9,10] However, from past experience, it is likely that it will only be a matter of time before the bacteria develop a mechanism of resistance to overcome any new antibiotic that is incorporated in bone cement. Therefore, ongoing research and industrial developments includes the use of combinations of antibiotics into PMMA bone cements, the so-called multidrug loading. Multidrug targeting is assumed not only to be more powerful, but also to prevent the emergence of resistant strains through synergistic action of two antibiotics at the same time.[11] Resistance potential is theoretically minimized by this technique because the antibiotics are typically from different antimicrobial classes, and different sites in the bacterial cell are targeted.

Multidrug loading of bone cements with two antibiotics is more difficult than in systemic delivery of two drugs, as the release kinetics of antibiotics from bone cement is dependent on many factors. Inclusion of a second antibiotic increases the porosity of bone cements[12] with an impact on the antibiotic release[13] and the mechanical strength of the cement.[14] A quantitative in vitro elution study showed an increased release of vancomycin and tobramycin when both added to a bone cement,[12] while mechanical properties remained acceptable, provided less than 1 g of antibiotic per 40 g of cement powder was added.[15–17]

Within the limited number of antibiotics available that are easily dissolved in an aqueous fluid and that can withstand the heat generated during the mixing of bone cement prior to use, fusidic acid and clindamycin are suitable candidates, besides gentamicin. In Europe there is one commercially available, multidrug-loaded bone cement (Copal; Merck, Darmstadt, Germany), containing gentamicin and clindamycin. A combination of gentamicin and clindamycin in bone cement has a theoretical antimicrobial effect on more than 90% of the bacteria common to infected arthroplasty cases.[14] Moreover, the release of gentamicin seems to be additionally favored by the release of clindamycin in this cement.[14]

The aim of this study is to investigate in vitro whether combinations of fusidic acid or clindamycin with gentamicin in a bone cement yields additional antimicrobial effects against a collection of 38 clinical isolates, including 16 gentamicin-resistant strains. Gentamicin-loaded bone cement was used as the control. In addition, six strains were selected from the collection of 38 clinical bacterial strains to investigate bacterial survival in a simulated prosthesis-related interfacial gap, with a geometry closely similar to the clinical situation and made out of the different antibiotic-loaded bone cements. A model simulating the in vivo interfacial gap,[18] existing between bone cement and bone or prosthesis was recently developed.[19] Studies on combinations of antibiotics included in bone cements using this gap model could be useful to estimate the clinical value of these combinations.

MATERIALS AND METHODS

Bone Cements

Three experimental antibiotic-loaded SmartSet® HV bone cements (DePuy CMW International Ltd., Cornford road, Blackpool Lancashire FY4 4QQ, United Kingdom) were used. Cements were commercially loaded with the mechanical safe maximum of 1 g antibiotic per 40 g powder: 1 g gentamicin base (G), a combination of 1/2 g gentamicin base and 1/2 g fusidic acid base (F) or a combination of 1/2 g gentamicin base and 1/2 g clindamycin base (C).

Sensitivity of Clinical Isolates to Different Bone Cements

Cement Disc Preparation

Cements were prepared by mixing the powdered methylmethacrylate with the liquid monomer in a bowl with a spatula. Manual mixing was done according to the manufacturer's instructions and resulted in liquid cement. The liquid cement was spread over a polytetrafluoroethylene (PTFE) mold ($200 \times 40 \times 3.2$ mm), containing holes of 6-mm diameter. The filled mold was

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manually pressed between two glass plates, covered with copier overhead film (Océ, MC 110, 's Hertogenbosch, The Netherlands) to facilitate removal after hardening, up to the time specified for final hardening. After 24 h the cement discs were pulled out of the mold and stored under dark, sterile conditions at room temperature. The total surface area of each disc was 1.2 cm^2, and one disc weighted about 100 mg. All procedures were carried out under sterile conditions.

Bacterial Strains for Modified Kirby-Bauer Test

A total of 38 bacterial strains (see Table 1) was used. The strains were isolated with extensive biomaterial culturing[20] from explanted joint prostheses from different patients with septic loosening. The susceptibility for gentamicin, clindamycin, and fusidic acid of these clinical strains is unknown, but they represent the wide spectrum of bacteria, which may be encountered after joint arthroplasty. The collection includes Gram-positive and Gram-negative aerobic and Gram-positive anaerobic bacteria. The distribution of the strains of each organism was chosen to represent their frequency of occurrence in clinical situations. Biomaterial-associated infections in orthopedic implants are often due to *Staphylococcus* species, while aerobic Gram-negative bacteria cause 10–20% of all deep infections and anaerobic bacteria are responsible for another 10–15%.[21,22] Thus, multiple strains of more frequently encountered strains such as *Staphylococcus aureus* and Coagulase-negative staphylococci (CNS) and individual strains of the less common pathogens, *Photobacterium logei* and *Enterococcus faecium*, were used.

The bacteria were cultured from cryopreservative beads (Protect Technical Service Consultants Ltd., United Kingdom) onto blood agar plates at 37°C in ambient air (except for the anaerobes) for 24 h and suspended in 0.9% saline to a concentration of 10^8 bacteria/mL. This suspension was used to inoculate Tryptone Soya Broth (TSB, Oxoid, Basingstoke, Great Britain) agar plates with a sterile cotton swab. Blood agar plates were used instead of TSB for two anaerobes, because these bacteria did not grow on TSB. The thickness of the agar was approximately 5 mm. Ten minutes after inoculation the bone cement discs were placed firmly in the center of each plate, and the plate was subsequently incubated aerobically or anaerobically for the anaerobes at 37°C. Zones of inhibition around the discs were measured after overnight incubation. The zone of inhibition was considered as the clear area around a disk in which bacteria were not able to grow. The diameter of each zone was measured in two directions and the mean zone of inhibition was calculated.

The size of the zone of inhibition is used to determine whether the bacteria are sensitive or resistant to the antibiotic(s) in the used bone cement discs. Sensitive strains possess a clear inhibition zone and larger diameters indicate a higher sensitivity. Sensitivity was recorded if a zone of inhibition of at least 12 mm was present. The absence of a zone of inhibition was taken as an indication of antibiotic resistance. No intermediate cases were seen. Occasionally a "ghost" zone existed between the areas of complete inhibition and full bacterial growth. This "ghost" zone is the result of partial inhibition, and was included in the measurement of the zone of inhibition. Samples that did not have a zone of inhibition were reported as NZ (no zone).

Bacterial Survival in an Interfacial Gap Model

Preparation of the Gaps

The physiological environment of a bone cement mantle has been mimicked by bone cement blocks with a 200-μm gap. The method for preparing these blocks is detailed elsewhere.[19] In short, the bone cement dough resulting from mixing the powder and liquid components was applied to a PTFE mold fitted with 200-μm wide stainless steel strips. After 24 h the blocks were removed from the molds and the strips. The yielded gap had a surface area of 0.6 cm^2 and a volume of 6 μL. Blocks were macroscopically examined, and those with visibly entrapped gas bubbles or other defects in proximity of the surface were discarded. Sterile precautions were observed throughout the entire procedure.

Bacterial Strains for Interfacial Gap Model

Three gentamicin-sensitive bacterial strains (CNS, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) together with three gentamicin-resistant CNS were used. These bacteria were selected from the strains used for the modified Kirby-Bauer test and indicated with a # in Table 1. All strains showed similar sensitivity to clindamycin and fusidic acid. The gaps were filled with 6 μL of a 1:10 dilution in TSB of a 24 h preculture of the strain in TSB. The preculture yielded a mean growth density for all bacteria of 2.1×10^8 cfus/mL, as determined by counting the number of colony forming units (cfus) after growth of serial dilutions on TSB agar plates. The inoculated gaps were incubated for 24 h at 37°C, after which the blocks were broken. The gap surfaces were scraped off and the scrapings were resuspended in 0.9% saline. After serial dilutions, TSB agar plates were used to quantify the number of cfus that had survived in the different gaps. Plates were incubated for 7 days to allow detection of slowly growing colonies after antibiotic exposure. A similar serial dilution and plating technique had been used to quantify the inoculum and the percentages of surviving bacteria with respect to the initial inoculum were calculated.

Table 1. Zones of Inhibition (mm) around Bone Cement Discs, Loaded with Only Gentamicin (G), Gentamicin and Fusidic Acid (F), or Gentamicin and Clindamycin (C) for 38 Clinically Isolated Bacterial Strains

	Diameter of the Zone of Inhibition (mm)		
Bacterial Strain	G	F	C
Gentamicin-sensitive			
Staphylococcus aureus	26	33	37
Staphylococcus aureus[#2]	20	30	34
Staphylococcus aureus	16	30	31
CNS[#1]	31	40	45
CNS	30	42	40
CNS	25	33	40
CNS	13	25	13
CNS	25	34	24
Streptococcus pyogenes gp A	14	17	32
Leuconostoc dextranicum	15	16	14
Micrococcus luteus	25	38	40
Corynebacterium	32	45	38
Pseudomonas aeruginosa[#3]	17	15	15
Pseudomonas aeruginosa	19	16	15
Pseudomonas diminuta	24	23	28
Photobacterium logei	26	25	23
Stenotrophomonas maltophilia	20	NZ-20[a]	NZ
Stenotrophomonas maltophilia	21	NZ-20[a]	NZ-18[a]
Propionibacterium acnes	25	16	60
Propionibacterium acnes[b]	20	18	59
Peptostreptococcus magnus	30	24	62
Peptostreptococcus magnus[b]	12	23	37
Gentamicin-resistant			
Staphylococcus aureus	NZ	NZ	NZ
CNS	NZ	36	41
CNS	NZ	30	34
CNS[#4]	NZ	36	39
CNS[#5]	NZ	35	37
CNS	NZ	18–43[a]	NZ
CNS	NZ	32	36
CNS	NZ	34	NZ
CNS	NZ	31	NZ
CNS[#6]	NZ	33	35
CNS	NZ	32	NZ
Streptococcus sanguinis	NZ	21	NZ
Enterococcus faecium	NZ	19	NZ
Alloiococcus otitis	NZ	26	NZ
Photobacterium logei	NZ	NZ	NZ
Comamonas acidovorans	NZ	NZ	NZ

NZ = no zone.
[a]Ghost-zone.
[b]On blood agar.
[#1–6]Used in model interfacial gap.

All experiments were carried out in triplicate with separately cultured strains, unless bacterial growth was fully absent on all three tested antibiotic-loaded bone cements in the first experiment in which case the experiment was only performed once. The differences between the survival percentage in gentamicin were compared with that in clindamycin and fusidic acid using Student's *t*-test for paired samples, because the inoculum size for each bone cement was identical in one run, but not between runs.

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RESULTS

Sensitivity of Clinical Isolates to Different Bone Cements

The zones of inhibition measured around bone cement discs loaded with gentamicin or combinations of gentamicin with fusidic acid or clindamycin are presented in Table 1. The absences of a zone of inhibition was taken as an indication of antibiotic resistance and, under this definition, bone cement loaded with gentamicin only had an antimicrobial activity against 58% of the strains isolated. This antimicrobial activity could be increased to 68% by addition of clindamycin. Bone cement discs loaded with the combination of gentamicin and fusidic acid had antimicrobial activity against 87% of the strains.

Gentamicin-Sensitive Bacteria

An additional antimicrobial effect from adding fusidic acid or clindamycin to gentamicin-loaded bone cement could be seen for most aerobic Gram-positive bacteria, whereas no additional effect could not be seen for the aerobic Gram-negative rods. Moreover, the two *Stenotrophomonas* strains showed adverse antimicrobial effects from adding fusidic acid or clindamycin to gentamicin-loaded cement. Remarkably, although anaerobes are theoretically resistant to gentamicin, in this study all anaerobes were sensitive for gentamicin-loaded bone cement, and additional antimicrobial effects could only be seen with the combination of gentamicin and clindamycin.

Gentamicin-Resistant Bacteria

The combination of fusidic acid and gentamicin in bone cement showed antimicrobial activity for 13 out of the 16 aerobic Gram-positive bacteria, which were resistant to gentamicin, whereas only six were affected by the combination of clindamycin and gentamicin.

Bacterial Survival in an Interfacial Gap Model

Bacterial survival in the gaps after 24 h of inoculation is presented in Table 2. The three gentamicin-sensitive strains were not able to survive in gaps in all three tested antibiotic-loaded bone cements. For the three gentamicin-resistant strains, there was a trend of more reduced survival in the bone cement containing gentamicin and clindamycin or fusidic acid compared with cement containing only gentamicin, but this trend could not be proven statistically significant at an acceptable level of $p < 0.05$. In one strain a limited increase in survival was seen with the combined use of fusidic acid and gentamicin.

DISCUSSION

The prophylactic use of antibiotic-loaded bone cements for the fixation of implants must be clearly distinguished from its therapeutic use in revision. Worrisome in the prophylactic application of antibiotic-loaded bone cements is the chance of introducing resistant strains. Hope et al.[23] found that the use of gentamicin-loaded bone cement in the primary arthroplasty was significantly associated with the emergence of gentamicin-resistant CNS in subsequent deep infection. The exact mechanism by which this antibiotic resistance evolves is not clear and different options have been reported, including

Table 2. Percentage Survival of Gentamicin Sensitive and Gentamicin-Resistant Bacterial Strains after 24 h Inoculation in a Gap in Antibiotic-Loaded Bone Cements

Bacterial Strain	Gentamicin Sensitivity	Survival (%)			*p*-value	
		G	*C*	*F*	*G vs. C*	*G vs. F*
CNS[#1]	S	NG	NG	NG	—	—
S. aureus[#2]	S	NG	NG	NG	—	—
Ps. aeruginosa[#3]	S	NG	NG	NG	—	—
CNS[#4]	R	0.154	0.006	0.265	0.09	0.15
CNS[#5]	R	0.008	0.003	0.000	0.42	0.22
CNS[#6]	R	0.106	0.009	0.009	0.19	0.19

NG = no growth.
The *p*-values as calculated with a two-tailed Student's *t*-test for paired samples are also given.

mutation and selection. Thomes et al.[24] stated that gentamicin-loaded bone cement has an optimum surface for bacterial colonization and prolonged exposure to gentamicin allows mutational resistance to occur. However, it is important to realize that antibiotics probably do not directly cause mutation; rather, they provide an environment favoring the natural selection of resistant strains[3,25] that arise spontaneously. The drug kills the sensitive bacteria, leaving behind those that can resist it. Thus, antibiotic-loaded bone cement may not technically cause resistance but allows it to happen by creating a situation where an already existing resistant variant can multiply and become the predominant bacterial population.

When gentamicin-loaded bone cement has been used in the primary arthroplasty, bacteria involved in these infections have probably survived the high gentamicin concentrations inside the interface between bone and bone cement and are likely to be gentamicin resistant. Subsequent use of gentamicin-loaded bone cement would therefore be less effective, while still carrying the risk of further selecting resistance. Therefore, gentamicin-loaded cement may not be appropriate for revision surgery if it has been used already in previous surgery.[24]

The choice of the two additional antibiotics used in this study can be rationalized. Clindamycin broadens the antimicrobial efficacy of gentamicin-loaded bone cement because it also covers anaerobic bacteria[14] and its susceptibility of bacteria adhering to PMMA has not been shown to change due to growth in the adhered state.[26] Fusidic acid has good efficacy against staphylococci, including strains resistant to other classes of antibiotics.[27] Moreover, fusidic acid has an excellent bone penetration,[28] and has therefore been suggested as a promising agent against bone infections. Fusidic acid is mostly given in combination with other antibiotics, because of the high spontaneous mutation rate for fusidic acid when given alone. Several authors have examined the properties of fusidic acid incorporated into bone cement, with conflicting results. Hill et al.[29] showed that fusidic acid, incorporated in Simplex bone cement was not active in vitro after 3 days, while gentamicin and clindamycin showed activity for 22 days and 56 days, respectively. Wahlig and Dingeldein[4] investigated the in vitro release of gentamicin, clindamycin, and fusidic acid from Palacos bone cement and found that only gentamicin and clindamycin were released in microbiologically significant amounts. In contrast, some studies showed that the in vitro antimicrobial activity of CMW cement containing fusidic acid persisted for 50 days.[30,31] One study, examining the in vitro activity of combined gentamicin and fusidic acid in CMW cement, demonstrated an enhanced inhibitory effect for the first 3 days that disappeared after 5 days.[31]

In this study, multidrug loading of acrylic bone cements was evaluated with respect to antimicrobial effects against a large variety of clinically isolates, all representing the strains that may be encountered after joint arthroplasty. The modified Kirby Bauer test showed an additional antimicrobial effect for both fusidic acid and clindamycin in combination with gentamicin. The combination of fusidic acid and gentamicin seems effective, however, against a larger number of clinical isolates than the combination of gentamicin with clindamycin, as can be seen from the antimicrobial spectrum of these combinations derived from the present results (see Fig. 1).

A modified Kirby-Bauer test, involving measurement of the inhibition zone around antibiotic-loaded bone cement discs on agar plates, was used in this study to investigate whether adding a second antibiotic has an additional antimicrobial effect. Because zone size is a function of the diffusion rate of the drug through the agar in addition to the drug's potency, smaller inhibition zones produced do not imply less inhibitory action. Additional factors influencing zone size include the drug's molecular weight and charge. Clindamycin and fusidic acid demonstrated inhibitory activity after mixing through bone cement, but quantification of their activity was not possible in this way. Therefore, we also tested the different bone cements in a simulated prosthesis-related interfacial gap. In previous reports, it was shown[32] that bacteria were able to grow and adhere on antibiotic-loaded bone cement samples with a large volume-to-area ratio. In a more clinically relevant situation, the volume-to-area ratio is very small, however, for which reason we developed our so-called "interfacial gap" model, in which the amount of fluid in between the surfaces to be colonized is small.[25] This model, simulating the in vivo interfacial gap, existing between bone cement and bone or prosthesis, offers an additional in vitro test that can be used to estimate the value of antibiotic additions to bone cement.[19,25] Hendriks et al.[19] demonstrated that concentrations up to 1000-fold the antibiotic resistance levels for most bacterial strains causing implant infection

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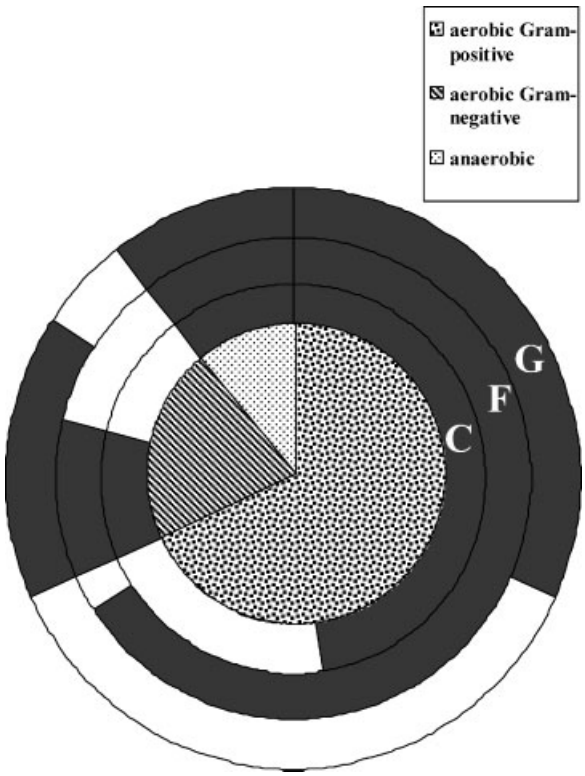

Figure 1. The antimicrobial spectrum of gentamicin-loaded bone cements with fusidic acid or clindamycin added against commonly infecting bacteria in joint implants (based on Table 1). The dark gray zones indicate antimicrobial activity and the different circles represent the different antibiotic combinations (G = gentamicin, F = gentamicin + fusidic acid, and C = gentamicin + clindamycin).

can be achieved in this more realistic in vitro model. Within 2 h, gentamicin concentrations in the interfacial gaps made in several commercially available gentamicin-loaded bone cements reached up to 2500–4000 μg/mL. Our study showed that despite the high gentamicin concentrations inside these gaps, concentrations could only kill the gentamicin-sensitive strains. We found a clear trend toward less bacterial survival in gaps made of bone cements with combinations of gentamicin and clindamycin or fusidic acid, because the gentamicin-resistant strains, able to survive for 24 h in a gap environment in gentamicin-loaded bone cement, are killed in a higher percentage if half of the gentamicin is replaced with fusidic acid or clindamycin.

Over the last 2 decades, the increasing incidence of methicillin-resistant staphylococci has caused significant clinical concern worldwide.[33] Methicillin resistance in staphylococci is also associated with resistance to several commonly used antimicrobial agents. Infections caused by resistant bacteria are associated with higher rates of hospitalization, greater length of hospital stay, and higher rates of illness and death.[34] With the increasing incidence of multidrug-resistant staphylococci in many countries the need for more active antistaphylococcal drugs has become apparent. Maple et al.[35] determined the antibiotic resistance patterns for more than 100 strains of methicillin-resistant *Staphylococcus aureus*. Resistance to gentamicin was recorded in more than 90% of the strains, whereas resistance to clindamycin and fusidic acid was respectively 66 and 12%. Determination of the in vitro susceptibility of 100 methicillin-resistant *S. aureus* and 100 methicillin-resistant coagulase-negative staphylococci showed that all strains were highly sensitive to fusidic acid.[36] In other words, fusidic acid has the potential to become an important antibiotic in the context of multidrug-resistant staphylococci. In our current study, fusidic acid in combination with gentamicin incorporated in bone cement appeared markedly effective against aerobic gentamicin-resistant cocci. The prominence of gentamicin-resistant cocci as the causative agent in infections in orthopedic joint implants[37] suggests that gentamicin-loaded bone cement with fusidic acid added can play a potentially important role in the prevention of these infections.

Although direct correlation between in vitro results and clinical performance is difficult, this study showed that gentamicin-loaded bone cements with clindamycin or fusidic acid added seem preferred formulations for prophylaxis in revision procedures, particularly when gentamicin-loaded bone cement was used in the prior procedure.

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