Clinical Microbiology

Different microbial biofilm formation on polymethylmethacrylate (PMMA) bone cement loaded with gentamicin and vancomycin

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ABSTRACT

We studied the in vitro effects of gentamicin and vancomycin alone and in combination added to polymethylmethacrylate (PMMA) cement specimens on the bacterial adhesion of multiresistant clinical isolates.

The PMMA specimens (discs) loaded with gentamicin (1.9%) or vancomycin (1.9%) or with a combination of the two were placed in Mueller-Hinton Broth inoculated with bacterial strains. After incubation, bacterial growth was determined by optical density (OD540) and sub-cultures. The biofilm PMMA-associated dye (crystal violet) was measured. Antibiotic concentrations in broth were determined by fluorescence polarisation immunoassay.

All antibiotic-loaded PMMA cement specimens released high, inhibitory concentrations of gentamicin and vancomycin. However, differences in strain growth and adhesion were recorded. The clinical isolates Met-R/Gent-R CoNS showed no adhesion to gentamicin-loaded specimens for 24 h; strains with Gent-Intermediate susceptibility exhibited growth after 48 h but reduced adhesion. Some Gent-R strains exhibited growth and adhesion to antibiotic-loaded specimens similar to controls (plain discs). Only the VRSA strain (Staphylococcus aureus 5/7) and Escherichia coli were able to grow and adhere to vancomycin-loaded specimens after 24 h of incubation. The specimens loaded with the gentamicin + vancomycin combination showed a synergistic inhibitory effect against all tested strains (no bacterial growth). The degree of bacterial adhesion to PMMA cement loaded with gentamicin or vancomycin may be reduced in spite of a normal growth rate and is different for the tested strains.

The effect of gentamicin and vancomycin on bacterial growth and adhesion to PMMA bone cement depends on the antibiotic concentrations, on the characteristics of each specific strain and on its ability to produce biofilm and adhere to antibiotic-loaded PMMA bone cement.

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1. Introduction

Polymethylmethacrylate (PMMA) bone cement is commonly used for the fixation of joint prostheses, as well as for the production of temporary prostheses, beads and hand-made or preformed spacers [1].

After implantation a biomaterial is covered by macromolecules and cells from the surrounding substrate/media. Bacterial adhesion depends on surface characteristics (roughness, charge, hydrophobicity, hydrodynamics, shape, chemical composition), surface conditions (antibiotic, host tissue, presence of other micro-organisms), and the biological environment (fluids, inflammation, host defenses, etc.) [2].

Among biomaterials, PMMA is considered the most prone to bacterial infection and PMMA bone cements also exhibit a substantial degree of porosity; nonetheless, it has been demonstrated that the presence of aminoglycosides (gentamicin, tobramycin) greatly reduces the adhesion capacity of bacteria compared to plain PMMA [3,4]. The combination with vancomycin seems to reduce or prevent the adhesion of aminoglycoside-resistant staphylococci [5].

However, the presence of bacteria also on antibiotic-loaded cement beads has recently been described albeit with sporadic frequency [6]. The amount of drug in antibiotic-loaded cement, the elution capacity and different properties of branded PMMA bone cement are factors responsible for the appreciable degree of variability of the antimicrobial effect and clinical outcome. Differences among commercial products, their utilisation in the clinical setting, the variability of the parameters and end-points adopted make it difficult to compare the results reported in the literature, both in vitro and in vivo, with the result that convincing, conclusive data are not available.
The most frequent isolates in orthopaedic prosthetic infections are Gram-positive cocci. Methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (CoNS) are proving more frequent. *Staphylococcus epidermidis* produces large-size biofilm more frequently than *S. aureus* [7], but not all *S. epidermidis* strains are capable of producing biofilm [7,8].

Antibiotic-loaded cements may prove beneficial in the treatment of these severe infections [9].

We studied the effect on growth inhibition and bacterial adhesion on polymerized PMMA bone cements loaded with gentamicin, vancomycin and gentamicin + vancomycin combination against different strains of multiresistant Gram-positive clinical isolates in vitro.

2. Materials and methods

2.1. Specimens

Discs measuring 10 mm in diameter, 1.5 mm in height, 0.24 g (range: 0.21–0.28 g) in weight and with a surface area of 204.1 mm² were prepared using polymethylmethacrylate (PMMA) bone cement (Cemex HP, Tecres) according to the manufacturer’s procedures.

Gentamicin (1.9%, gentamicin sulphate, Shanghai Fourth Pharmaceutical, China) and vancomycin (1.9%, vancomycin hydrochloride, Alpharma ApS) alone and in combination were mixed with PMMA copolymer powder before addition of the liquid polymer under laminar flow. The following batches were prepared: lot 1 - gentamicin-loaded cement; lot 2 - vancomycin-loaded cement; lot 3 - gentamicin plus vancomycin-loaded cement and lot 4 - plain bone cement (control). The mean amount of gentamicin and vancomycin alone in each disc was 4.5 mg (4.5 ± 4.5 mg in the combination).

Specimens were sterilized with ethylene oxide according to ISO 11135:1994 for medical devices.

2.2. Bacterial strains

Bacterial strains were multiresistant clinical isolates obtained from Intensive Care Unit inpatients and from catheter infections, kindly provided by the Microbiology Department of the Verona University Hospital. The following strains were studied: methicillin-susceptible and gentamicin-susceptible (MS-GS) *S. aureus* 3A10; methicillin-resistant and gentamicin-resistant (MR/GR) *S. epidermidis* 8/28, *Staphylococcus haemolyticus* 8/28 and *S. epidermidis* 3/2; methicillin-resistant and gentamicin-intermediate (MR/GI) *S. epidermidis* 137/25 and *Staphylococcus hominis* 126/26; methicillin-resistant, vancomycin-resistant and gentamicin-resistant (MRSA,VRSA,GRSA) *S. aureus* 5/7, a high biofilm producer. Gentamicin-susceptible (G-S) *Escherichia coli* 7A27 was included as representative of Gram-negative strains and for gentamicin antimicrobial activity evaluation.

Resistance of the staphyloccocal strains was determined according to international standard methods (Clinical and Laboratory Standard Institutes, CLSI) [10]. Resistance to gentamicin was defined by MIC₉₀ > 32 mg/L; gentamicin-intermediate by MIC₉₀ = 8.0 mg/L, and resistance to vancomycin by MIC₉₀ > 4.0 mg/L for tested strains.

The effect of the gentamicin + vancomycin combination, determined by the checkerboard technique, was synergistic, the fractional inhibitory index being 0.25.

2.3. Biofilm evaluation

Bacterial adhesion was determined using the method described by O’Toole and Kolter [11] and Peeters et al. [12] with appropriate modifications.

Disc specimens of each group were placed singly in a glass flask containing 50 ml of sterile Mueller–Hinton Broth. The medium was inoculated with 10 microliters of bacterial strain (5 × 10⁶ CFU/ml, final concentration) by overnight culture at 37 °C.

A plain cement specimen placed in the medium without inoculum and processed along with other specimens constituted the absolute control (blank). Three specimens were utilized for each group for each strain in each experiment (total 400 specimens).

After incubation at 37 °C for 18 h, bacterial growth was determined by spectrophotometry (OD₅₄₀ nm, vertical reading, Bioscreen, Labsystem). In the case of absent or doubtful bacterial growth, the incubation at 37 °C was prolonged, recording bacterial growth for 72 h.

In parallel, sub-cultures in Brain-Heart Agar were performed to evaluate the correspondence between optical density (OD) and bacterial viability.

After bacterial growth, each specimen was removed from the flask, rinsed with sterile water, vortexed (twice) to eliminate bacterial planktonic forms, and then stained with 2 ml of crystal violet (1%) for 30 min under agitation [11,12].

Evaluation of the colour of the specimens was first performed utilizing an arbitrary scale ranging from 0 to ++ (++ – no growth; ++ – doubtful; + – poor; ++ – good; +++ – intense; ++++ – very intense).

Each disc was then rinsed with saline, immersed in ethanol (2 ml, 95% v/v) for 30 min with agitation to solubilize the adherent stained bacteria. The higher the number of bacteria in the solution, the more intense will appear the staining of the solution. The coloured solution, diluted with sterile water (2 ml), was quantified spectrophotometrically (OD₅₄₀ nm) [11,12].

The values obtained from antibiotic-loaded PMMA specimens were compared with those obtained from plain PMMA cement, and the bacterial adhesion was determined for each strain.

All tests were performed in duplicate, in different (2–4) experiments.

Results are given as mean values ± SD. The results were analyzed statistically using Student’s t-test.

2.4. Determination of antibiotic concentration

The concentrations of antibiotics released from each specimen were determined in the broth at the end of the experiment, i.e. at 24, 48 and 72 h according to bacterial growth.

Table 1

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Gentamicin</th>
<th>Vancomycin</th>
<th>Gentamicin + Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> 3A10</td>
<td>10.5 ± 1.7</td>
<td>5.0 ± 0.7</td>
<td>G 13.3 ± 0.5</td>
</tr>
<tr>
<td>GS/MS</td>
<td></td>
<td></td>
<td>V 6.4 ± 0.9</td>
</tr>
<tr>
<td><em>S. aureus</em> 5/7</td>
<td>8.2 ± 1.0</td>
<td>4.8 ± 0.9</td>
<td>G 11.6 ± 2.2</td>
</tr>
<tr>
<td>MRSA/VRSA/GRSA</td>
<td></td>
<td></td>
<td>V 6.5 ± 0.9</td>
</tr>
<tr>
<td><em>S. epidermidis</em> 8/28</td>
<td>9.7 ± 1.7</td>
<td>5.2 ± 0.9</td>
<td>G 11.5 ± 1.9</td>
</tr>
<tr>
<td>MR/GR</td>
<td></td>
<td></td>
<td>V 7.1 ± 0.9</td>
</tr>
<tr>
<td><em>S. hominis</em> 126/26</td>
<td>8.1 ± 0.4</td>
<td>3.8 ± 0.6</td>
<td>G 21.2 ± 1.9</td>
</tr>
<tr>
<td>MR/GR</td>
<td></td>
<td></td>
<td>V 9.4 ± 0.6</td>
</tr>
<tr>
<td><em>S. epidermidis</em> 137/25</td>
<td>7.5 ± 1.0</td>
<td>4.2 ± 1.1</td>
<td>G 11.9 ± 2.5</td>
</tr>
<tr>
<td>MR/GR</td>
<td></td>
<td></td>
<td>V 5.8 ± 1.5</td>
</tr>
<tr>
<td><em>S. haemolyticus</em> 8/28</td>
<td>9.0 ± 2.7</td>
<td>4.8 ± 1.2</td>
<td>G 12.2 ± 3.3</td>
</tr>
<tr>
<td>MR/GR</td>
<td></td>
<td></td>
<td>V 4.7 ± 0.3</td>
</tr>
<tr>
<td><em>S. epidermidis</em> 3/2</td>
<td>9.2 ± 0.8</td>
<td>5.0 ± 0.02</td>
<td>G 11.3 ± 3.8</td>
</tr>
<tr>
<td>MR/GR</td>
<td></td>
<td></td>
<td>V 7.4 ± 0.5</td>
</tr>
<tr>
<td><em>E. coli</em> 7A27</td>
<td>9.9 ± 0.2</td>
<td>5.9 ± 0.01</td>
<td>G 11.9 ± 0.6</td>
</tr>
<tr>
<td>G-S</td>
<td></td>
<td></td>
<td>V 6.5 ± 0.03</td>
</tr>
</tbody>
</table>
The concentrations of gentamicin and vancomycin released from PMMA specimens were determined by fluorescence polarization immunoassay (FPIA, TDx, Abbott). The standardized procedures have been described in detail [13,14].

3. Results

The results of bacterial growth and adhesion varied according to the characteristics of the strain, its susceptibility to gentamicin and vancomycin, and the concentration of antibiotic released by the antibiotic-loaded PMMA discs.

No growth was recorded in the absolute control flasks (plain cement without inoculum).

All tested bacterial strains were able to adhere to plain PMMA bone cement specimens (controls).

All specimens released high, effective concentrations of antibiotics alone or in combination although a substantial degree of variability was recorded among the discs. The mean concentration ranges of gentamicin and vancomycin alone were 7.5–10.5 mg/l and 3.8–5.9 mg/l, respectively; similar amounts of each antibiotic were released from the combination (Table 1). The two antibiotics in combination exerted a bactericidal effect (no growth even after 72 h).

Disc specimens loaded with gentamicin and vancomycin alone showed different inhibitory effects according to the susceptibility of each strain to the antibiotics. Moreover, disc specimens loaded with the combination of gentamicin and vancomycin showed a bactericidal effect and synergistic activity against all multiresistant strains.

We recorded substantial variability in drug antimicrobial activity and adhesion capacity among the tested strains. The results are summarized in Table 2. *S. aureus* 3A10 GS-MS showed poor growth (0.35 ± 0.03 OD, mean ± SD) in the first 24 h. After 48 h the growth of *S. aureus* 3A10 was evident (0.565 ± 0.014 OD) but reduced in comparison to controls (0.805 ± 0.056 OD); the adhesion was similar to controls. Gentamicin was released at concentrations (10.5 mg/L) higher than the MIC for *S. aureus* 3A10. Vancomycin, both alone and in combination with gentamicin, inhibited bacterial growth for the duration of the study.

*S. aureus* 5/7 VRSA/MRSA/GRSA showed good growth and adhesion in the presence of gentamicin alone. In the presence of vancomycin alone growth occurred only after 48 h (0.591 ± 0.303 OD), while the adhesion was lower (0.171 ± 0.018 OD) than controls (plain cement) and remained low after 72 h. The gentamicin + vancomycin combination inhibited growth and adhesion for 72 h.

Strains with intermediate resistance to antibiotics (i.e. *S. epidermidis* 137/25) were inhibited by high concentrations of gentamicin (9.7 ± 1.7 mg/L, mean ± SD). Vancomycin and the combination inhibited bacterial growth more strongly than gentamicin alone for 48 h.

*S. hominis* 126/26 MR-GI showed bacterial growth after 24 h in the presence of gentamicin, but significantly reduced adhesion (0.182 ± 0.024 OD, p = 0.0007). Vancomycin, eluted at low concentrations (3.8 ± 0.6 mg/L), allowed good bacterial growth (1.110 ± 0.034, OD), but also in this case adhesion was significantly reduced (0.189 ± 0.028 OD, p = 0.0001) after 24 h. The combination exerted a distinct bactericidal effect.

*S. epidermidis* 8/28 MR-GR and *S. haemolyticus* 8/28 MR-GR showed growth and adhesion after 24 h equivalent to the respective controls in the presence of gentamicin and vancomycin alone. The combination inhibited the growth and the adhesion of both strains.

*S. epidermidis* 3/2 MR-GR showed complete growth (0.823 ± 0.016 OD) in the presence of gentamicin but low adhesion after 24 h. Vancomycin determined poor growth (0.220 ± 0.075 OD) and significantly low adhesion (0.146 ± 0.005 OD, p < 0.0005) in comparison to plain cement (0.213 ± 0.019 OD) after 24 h. The combination inhibited bacterial growth for 72 h.

The amounts of gentamicin (ranging from 7.5 mg/L to 10.5 mg/L) released from specimens were enough to inhibit bacterial growth of strains susceptible to gentamicin, such as *E. coli* 7A27 during the first 24 h. *E. coli* 7A27 G-S showed results similar to those obtained for *S. aureus* 3A10: in the presence of gentamicin bacterial growth was observed only after 48 h with significantly low adhesion (0.162 ± 0.07 OD, p < 0.05). Vancomycin alone did not inhibit the growth of *E. coli* 7A27. The gentamicin + vancomycin combination exerted a synergistic effect with 72-hour inhibition of bacterial growth.

The gentamicin + vancomycin combination inhibited the bacterial growth of all the strains studied, inclusive the multi-resistant VRSA/MRSA/GRSA *S. aureus* strain.

4. Discussion and conclusions

Adhesion of pathogenic bacteria to the surface of PMMA bone cement is considered to be the critical event in the development of infection during total hip arthroplasty [15,16]. Antibiotic-loaded PMMA cements may prove beneficial in the treatment of these severe infections [9]. Addition of antibiotics to methylmethacrylate cement with demonstrable elution over a period of time has been shown to be effective in the management of established...
periprosthetic infections, reducing the risk of infection and the associated risk of revision in primary total hip replacement [17].

Our results demonstrated that all PMMA cement specimens loaded with gentamicin, vancomycin and a combination of the two released effective concentrations of antibiotics.

Gentamicin and vancomycin alone were inhibitory against susceptible and intermediate-susceptible bacteria during the first 24–48 h. The two drugs, however, exerted different inhibitory activity on bacterial growth and adhesion, according to the characteristics of the strains concerned.

Vancomycin-loaded specimens inhibited bacterial growth and reduced bacterial adhesion. Bacterial adhesion of most strains was also reduced in the presence of low vancomycin concentrations.

The inhibitory activity of gentamicin against a number of strains of coagulase-negative methicillin-resistant staphylococcal clinical isolates was maintained for 24 h, followed by growth over the next 48 h. Strains with intermediate susceptibility to gentamicin showed normal growth after 48 h but reduced adhesion, while some gentamicin-resistant strains showed growth and adhesion similar to controls. Our results are in line with the data obtained by Dunne et al. [18], who demonstrated that the incorporation of additional gentamicin in bone cement reduced or prevented the colonization of S. aureus and S. capitis strains at 6 and 24 h, but none of the bone cements tested containing additional gentamicin (1–4 g) prevented colonization by these two strains at 48 and 72 h.

High concentrations of antibiotics usually exert a local inhibitory effect. Adhesion is possible when bacteria are not killed by adequate concentrations of antibiotics. However, in a number of prosthetic human infections the micro-organisms may colonize the cement but not the periprosthetic tissue, as in two-stage revision infections, when bacteria are isolated in the removed beads [19]. In a few cases micro-organisms were isolated only in tissue after bead removal, while in the presence of persistent infection, the micro-organisms were isolated both in beads and in tissue [19].

The microcolonies in cement beads or in cement for prosthesis fixation are an expression of different problems. The presence of bacteria on PMMA bone cement loaded with antibiotics utilised for prosthesis fixation denotes a risk of prosthetic infection as well as of increased resistance, while the beads used in two-stage revision infections are removed after a short to medium period of implantation, i.e. along with bacteria, if present on the surface of the beads [20].

The gentamicin + vancomycin combination exerted a synergistic bactericidal effect, even against multiresistant Gram-positive micro-organisms, such as S. aureus 5/7 (VRSA), a high biofilm producer.

Bacterial growth and adhesion differ according to: 1) strain characteristics; 2) gentamicin and vancomycin susceptibility and 3) the antibiotic concentration released by specimens.

These results obtained in vitro are useful for studying and understanding the differences in bacterial adhesion to PMMA bone cement loaded with antibiotics and for interpreting the clinical results. In vivo conditions, however, may mean that unpredictable interactions with the host need to be taken into account, as well as measures such as surgical debridement, systemic antibiotic therapy, etc.

In conclusion, the anti-adhesion effects of antibiotic-loaded PMMA cement depend on the characteristics of the micro-organism and its ability to adhere to antibiotic-loaded surfaces along with high drug concentrations. Bacterial adhesion was low in samples undergoing the highest antibiotic elution.

In addition to the susceptibility or resistance of bacterial strains to antibiotics, other factors such as the ability of strains to adhere to PMMA, or different production of biofilm and/or the presence of other biological products should be considered when assessing the ultimate antimicrobial effects of antibiotic-loaded cement.

References


