Evaluation of in vitro serial antibiotic elution from meropenem-impregnated polymethylmethacrylate beads after ethylene oxide gas and autoclave sterilization

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Keywords
Antibiotic-impregnated polymethylmethacrylate beads, meropenem, PMMA beads, polymethylmethacrylate, sterilization

Summary
Objectives: To determine the elution properties of meropenem and to compare the elution of meropenem-impregnated polymethylmethacrylate (PMMA) beads without sterilization (P-M-C) to those sterilized with steam (P-M-A) and ethylene oxide gas (P-M-EO).

Methods: A commercial bead mould was used to produce four groups of beads: one group without antibiotic (negative control), and three groups of meropenem-impregnated beads: P-M-C, P-M-A, and P-M-EO. The beads were placed in a phosphate buffered solution and eluent samples were collected. Concentrations of the antibiotic in eluent samples from the two sterilized groups and the control beads were determined using a microbiological assay at 1, 3, 6 and 12 hours and at 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days.

Results: The microbiological assay resulted in no zone of inhibition at all time periods for the P-M-A samples and the samples of PMMA without antimicrobial. The meropenem concentration on the eluent remained above 4 mcg/ml for 15 days in the P-M-C group and until day 18 for P-M-EO group. There was no statistical difference in AUC0-∞ (p < 0.318), however significance did occur for MRT (p < 0.005) when comparing P-M-C and P-M-EO with the later being higher.

Discussion: The meropenem incorporated in the PMMA beads eluted effectively and gradually decreased after the 24 hour peak, but remained above the concentration level of 4 mcg/ml for 15 days in the P-M-C group and until day 18 for P-M-EO group. Ethylene oxide does not adversely affect meropenem’s elution from PMMA beads.

Introduction
Osteomyelitis in small animals is most commonly post-traumatic and bacterial in aetiology. Infections are acquired by direct inoculation, extension from soft tissue infections or by haematogenous spread (1). The bacteria that are most frequently responsible for osteomyelitis in small animals are Staphylococcus spp, Streptococcus spp, and Gram-negative aerobic bacteria (1, 2). Treatment of osteomyelitis often includes surgical debridement of devitalised bone, lavage, fracture stabilisation, grafting of bone deficits, and systemic administration of antibiotic medications for periods ranging from four weeks to several months. A long course of intravenous antibiotic drug administration is the best method to reach therapeutic concentrations at the infection site, but this can be cost prohibitive in veterinary medicine. In addition, the attainment of adequate antibiotic drug concentrations is not always possible due to reduced blood supply at the infection site and the potential for systemic toxicity (3, 4).

The local implantation of antibiotic-impregnated polymethylmethacrylate (PMMA) for treatment of orthopaedic infections and wound infections, as well as for prophylaxis in joint arthroplasty has gained popularity due to the ability to attain therapeutic concentrations of antibiotic agents in the tissue while avoiding adverse effects from systemic toxicity (5, 6). Local therapy also allows use of antibiotic agents that may be otherwise cost prohibitive for long-term systemic administration (7). Antibiotic tissue concentrations in wounds treated locally with antibiotic-impregnated PMMA beads can be as much as 20-times the concentrations obtained in serum with systemic administration (8). The increased local antibiotic drug concentration may also broaden the spectrum of susceptible organisms (8). Antibiotic drug elution is biphasic, with a fast elution rate within hours to days after implantation, and a slow continuous rate in the weeks to months after implantation (8, 9). Good elution characteristics from PMMA have been reported for many antimicrobial agents, including aminoglyco-
sides, β-lactams, metronidazole, and macrolides (3, 6, 10). The surgeon has three options for bead manufacture; these include commercially manufactured gentamicin-impregnated PMMA beads and mould-made or hand-rolled beads. Prefabrication of antibiotic-impregnated PMMA beads reduces surgery time and cost, and it also results in a more consistent product (5). The ability to sterilize antibiotic-impregnated PMMA beads using ethylene oxide gas or autoclaving would allow for better accessibility and wider use by veterinary practitioners. The prefabrication and ethylene oxide gas sterilization of antibiotic-impregnated PMMA beads for ‘off the shelf’ availability of one year has been reported, but there have not been any commercial products approved for use in the United States (11, 12).

The widespread use of antibiotic drugs has resulted in an increased prevalence of resistant opportunistic pathogens in both referral and general veterinary hospitals (13). Because of the limited selection of antimicrobials for these multi-drug resistant infections, newer generation, broad-spectrum antibiotics, such as meropenem, are being used more frequently in veterinary medicine. Meropenem is an intravenous β-lactam antibiotic drug that belongs to the carbapenem subgroup (14). It is highly resistant to degradation by β-lactamases, cephalosporinases, and it is also stable to dehydropeptidase-I (14). The spectrum of activity of meropenem includes most Gram-positive and Gram-negative bacteria with the strongest affinities for Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus (14). Meropenem exhibits in vitro minimum inhibitory concentration (MIC<sub>90</sub>) and breakpoint sensitivity of 4 mcg/ml or less for most organisms including the organisms previously mentioned and anaerobes such as Bacteroides spp., Clostridium spp., and Fusobacterium spp. (14). Though there have been reports of bacterial resistance to antibiotic drugs in the carbapenem class in humans, to our knowledge there are no documented reports of bacterial resistance in veterinary medicine (14).

The ideal antibiotic agent for incorporation in PMMA should be bactericidal, broad-spectrum in activity, effective at low concentrations, highly water soluble, hypoallergenic, biocompatible, non-toxic for tissue, and stable at temperatures up to 100 °C (7). In addition, following implantation as an antibiotic-impregnated PMMA bead, it should not adversely affect the mechanical properties of PMMA, and result in low serum concentrations, but high concentrations in the target tissue (7). Based on these required characteristics, meropenem appears suitable for incorporation in PMMA cement.

To our knowledge there have not been any reports published that describe the evaluation of in vitro elution characteristics of meropenem-impregnated PMMA beads. The objective of this study was to evaluate the effects of sterilization by ethylene oxide gas or autoclaving on the elution properties of meropenem from PMMA beads.

Materials and methods

A commercially manufactured cast metal polytetrafluoroethylene coated bead mould was used to make beads from radio-opaque PMMA cement as described previously (5). Each mould produced one chain of 25 beads that were 6.4 mm in diameter. A biocontainment hood with a sterile laminar flow environment was disinfected with 70% ethanol and then exposed to ultraviolet light for 30 minutes prior to bead preparation. The commercial grade meropenem powder was combined with sterile PMMA powder at a ratio of 1:5 (4 g antibiotic:20 g PMMA) in a plastic bowl and thoroughly mixed with a wooden tongue depressor for two minutes using an aseptic technique (5, 15). The powder was then divided into four equal, 6 g portions (1 g antibiotic, 5 g PMMA) utilising a laboratory scale, and placed into specimen cups. 2.5 ml of the sterile liquid monomer was mixed with 6 g of the powder mixture, then stirred for 30 seconds and placed into two 10 ml syringes. A 0.644 mm diameter stainless steel cerclage wire was placed along the slot inside the bead mould and the mixture was injected into each hole of the bead mould with gentle pressure. The beads were allowed to harden for 30 minutes, then removed from the bead mould, placed in a sterile tube and allowed to harden over 24 hours at room temperature (68–70°F). This process was repeated four times to make five strands with a total of 125 beads, of which 120 were used. After 24 hours, the bead strands were cut into strands of five beads each. Negative control beads were made in the same fashion without the addition of meropenem.

Forty beads were placed in an autoclave (P-M-A) and sterilized for 15 minutes at 121°C. Forty beads underwent gas sterilization using ethylene oxide (P-M-EO) for three hours at 48.9°C. The final 40 beads containing meropenem were kept in sterile conditions, but did not undergo sterilization (P-M-C). These beads served as a positive control group. Three groups of 40 PMMA beads without meropenem were treated in the same fashion with autoclave (P-A), ethylene oxide (P-EO) or no sterilization (P-C) and these served as negative controls.

The process for evaluation of the beads consisted of placing one strand of five beads in individual test tubes (8 total test tubes per group) with 5 ml of sterile phosphate buffered saline (PBS) solution at pH 7.4. The beads were maintained at normal body temperature (37°C) and subjected to constant horizontal agitation at 15 cycles per minute (15). The PBS from experimental and control beads was sampled by complete evacuation of the 5 ml eluent fluid and replacement with another 5 ml of fresh PBS after sampling. The eluent samples were placed in 7ml plastic vials and stored at −80°C until assayed. Concentration of the antibiotic drug in eluent samples from the two sterilized groups and the control beads was determined using a microbiological assay at 1, 3, 6 and 12 hrs and at 1, 3, 6, 12 and 24 hours.

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a Septopal®; Kulzer, Wehrheim, Germany
b Antibiotic bead mold: University of Vermont, Instrument & Model Facility, Burlington, VT, USA
c Simplex®; P: Howmedica Inc, Rutherford, NJ, USA
d Labconco Purifier Class II Biosafty Cabinet: LABCONCO Corporation, Kansas City, MO, USA
emERM®; AstraZeneca Pharmaceutical LP, Wilmington, DE, USA

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1 Steris® Amsec® Century® SV-148H Prevac Steam: Steris Corporation, Mentor, OH, USA
2 Steris® Amsec® Eagle® 3017 EO Sterilizer: Steris Corporation, Mentor, OH, USA
The disc diffusion method was performed as previously described in other studies (17). If the concentration of meropenem exceeded the highest standard curve, the eluted samples were diluted with PBS before testing and adjusted accordingly.

*Kocuria rhizophila*, formerly classified as *Micrococcus luteus* ATCC 9341, was cultivated on Antibiotic Medium No. 1 agar and stored at −80°C. It was thawed and inoculated on a blood agar Petri dish 24 hours before the bioassay test and was kept in an incubator at 37°C until used the next day. *Kocuria rhizophila* has been previously used to assay a variety of antibiotics and fungicide residues (18–21). An inoculation system was used to prepare standardized suspensions adhering to the standards set by the American National Committee for Clinical Laboratory Standards. This inoculation system achieves the desired inoculum density, equivalent to the 0.5 McFarland turbidity standard. All eluent samples were thawed at room temperature and agitated before dilution or application to the blank paper discs.

An antibiotic medium was prepared following the manufacturer recommendations, autoclaved and poured at 225 ml per Nunc plate in a biocontainment hood, allowed to cool and refrigerated until used. Each Nunc plate was inoculated with *Kocuria rhizophila* using the Kirby-Bauer method, and all the elution samples and quadruplets of the standard curves were randomised by drawing sample numbers from a box. Each plate had duplicate internal standards of meropenem 16 mcg/ml that were used to adjust for interplate variability in zone size. Concentrations reported below the lower limit of the standard curve are due to division by a dilution factor and not extrapolation below the lower limit. The plates were allowed to dry after inoculation for 10 minutes and 24 6-mm diameter blank paper discs in duplicates were applied on the surface of the agar plate equidistant from each other using a sterile technique, and 20 mlc of the designated sample was applied to each disk and allowed to dry for 15 to 20 minutes before inverting the plate. The plates were incubated at 37°C for 24 hours. The zone diameters of the growth inhibition were measured using an electronic digital calliper 24 hours after inoculation and results were recorded for analysis. Areas of inhibition that were faint or less than the diameter of the disc were recorded as ‘no zone’ or ‘not reliably measured’. The unknown released meropenem concentration for the *in vitro* samples was determined by comparison of their respective zone size means with the standard curve (22).

The zone of inhibition is directly associated with drug response concentrations. As the concentration of the drug increases, the zone of inhibition should increase if the organism is susceptible. This linear relationship between the diameters of the zone of inhibition and the concentration of the drug allows use of the assay as a method for quantification of a drug.

### Statistical analysis

A pharmacokinetic analysis was performed by converting the concentration of eluted drug versus time into rate of elution versus time for each sample (23, 24). A model-independent pharmacokinetic analysis using statistical moment theory was performed using established equations based on area-under-the-curve calculations (25). The area under the curve from baseline to infinity (AUC∞) represents the total meropenem present in the eluted sample over time. The mean residence time from time zero to infinity (MRT∞) evaluates the behaviour of all the meropenem molecules in the dose, from the time that it was administered to the time that it was eliminated (41, 42). In order to calculate AUC∞ and MRT∞ parameters, the terminal rate constant (λz) was determined by simple linear regression of all non-zero data points beyond 48 hours.

The analysis of repeated measures data was performed by using procedure MIXED with the REPEATED statement on commercial computer software. The SUBJECT was TUBE (TREATMENT). The solution in each tube was modelled as subject, treatment (BIOASSAY) as between subject, TIME as the categorical variable, and AR (1) (autoregressive of order 1) covariance structure to specify the property of correlation of each tube being larger for nearby by time point than distant time point. Statistical analysis was performed using the concentration of drug eluted versus time and the rate of elution versus time. A one-way ANOVA was used for comparison of the pharmacokinetic parameters. The eight test tubes per group were sampled and analysed. The level of significance was set at p < 0.05.

### Results

None of the P-M-A samples or the negative control samples without meropenem (P-A, P-EO, P-C) resulted in any zone of inhibition at any of the time points for the microbiological assay. Due to the loss of antibacterial activity for P-M-A samples, statistical comparison to the other treatments was not possible.

Based on Cartesian and semi-log plots, it was determined that the elution for the P-M-C and P-M-EO samples followed a first-order process (23, 24). The pharmacokinetic moment parameters are shown in Table 1. The MRT∞ for P-M-EO was significantly higher than for P-M-C (p < 0.005), implying that meropenem had a higher total elution and drug persistence when treated with gas sterilization (26). The difference between P-M-C and P-M-EO in the AUC∞ was not significant (p < 0.318).

A mean cumulative drug elution for P-M-C and P-M-EO during 30 days totalled 3,210 mcg and 3,397 mcg, respectively. This represents 2.6% ± 0.4% (±SD) of

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**References**

1. Antibiotic Medium No #1: Alpha Biosciences, Inc, Baltimore, MD, USA
2. BBL™ Prompt™ inoculation system: Beckton Dickinson Microbiology Systems, Sparks, MD, USA
3. DIFCO™ antibiotic medium 11: Beckton, Dickinson and Company, Sparks, MD, USA
4. Thermo Scientific Nunc 245mm Square BioAssay Dishes: Fisher Scientific, Houston, TX, USA
5. BBL™ blank paper discs: Becton Dickinson Microbiology Systems, Sparks, MD, USA

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the total initial amount incorporated in the beads for P-M-C and 2.9% ± 0.2% for P-M-EO. This finding is in accordance with previously reported antibiotic elution percentages from PMMA ranging from 2.3% to 11% for penicillins, cephaloridine, clindamycin, sodium fusidate and gentamycin (27). The 24 hours elution for P-M-C and P-M-EO were 77.6% ± 4.6% and 66.6% ± 5.3%, respectively, of the total elution. The elution rapidly declined during the remaining time period with a total elution for P-M-C and P-M-EO of 22.4% ± 4.6% and 33.4% ± 5.3%, respectively, of that total drug elution (Table 2). The meropenem elution followed a biphasic pattern with a rapid elution during the first six hours when it reached a plateau, and a slower decline during the slow phase of the elution until the meropenem was not detectable (Fig. 1). The eluent remained above the breakpoint sensitivity limit of 4 mcg/ml for 15 days in the P-M-C group and until day 18 in the P-M-EO group. A breakpoint sensitivity limit designates the transition concentration between probable efficacy versus likely resistance to an antibiotic (22). Statistical analysis of least squares means for the mean concentrations of meropenem showed a significant difference between P-M-C and P-M-EO at 6, 12, and 24 hours and at two, three, six and nine days. The differences at one and hours and at 12, 15, 18 and 22 days were not significant. Statistical analysis of least squares means for the buffer concentrations showed a significant difference between P-M-C and P-M-EO at the baseline and at three hours. The differences at 9, 12, 15, 18, 21, and 24 hours and at 5, 10, 15, 20 and 25 days were not significant.

Table 1. Pharmacokinetic moment parameters (mean ± SD) of elution of meropenem from polymethylmethacrylate beads that were not sterilized (P-M-C) or sterilized by ethylene oxide gas (P-M-EO). The area under the curve from baseline to infinity (AUC∞) represents the total meropenem present in the eluted sample over time. The mean residence time form baseline to infinity (MRT∞) evaluates the behaviour of all the meropenem molecules in the dose, from the time that was administered to the time that was eliminated. λz is the first-order rate constant of the terminal elution phase. Half-life (t1/2) was derived by dividing 0.693 by the terminal rate constant. The standard deviation (SD) of the rate constant reflects the variability of the half-life. * Denotes a value that has statistically significant difference.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P-M-C</th>
<th>P-M-EO</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC∞ (mcg/ml)</td>
<td>3390.0 ± 593.15</td>
<td>3631.6 ± 288.76</td>
</tr>
<tr>
<td>*MRT∞ (hours): Mean residence time from time zero to infinity (evaluates the behaviour of all the meropenem molecules in the dose)</td>
<td>20.7 ± 4.19</td>
<td>27.6 ± 3.67</td>
</tr>
<tr>
<td>λz (hour⁻¹): The terminal rate constant</td>
<td>0.012 ± 1.253</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>t1/2 (hours) [Half life]</td>
<td>57.2</td>
<td>51.8</td>
</tr>
</tbody>
</table>

P-M-C: Meropenem-impregnated beads kept in sterile conditions, but did not undergo sterilization.
P-M-EO: Meropenem-impregnated beads that underwent gas sterilization using ethylene oxide.

Table 2. Concentration of meropenem eluted during the first and second elution phases and total elutions for polymethylmethacrylate beads that were not sterilized (P-M-C) and sterilized by ethylene oxide gas (P-M-EO).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total meropenem elution in 24 hrs (mcg/ml)</th>
<th>% Meropenem elution in 24 hrs</th>
<th>Total meropenem elution in 2nd phase (mcg/ml)</th>
<th>% Meropenem elution in 2nd phase</th>
<th>Total meropenem elution in 30 days (mcg/ml)</th>
<th>% Meropenem elution incorporated into 5 beads (mcg/ml)</th>
<th>% Meropenem elution in 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-M-C</td>
<td>2700.4</td>
<td>77.8 ± 4.6</td>
<td>768.9</td>
<td>22.2 ± 4.6</td>
<td>3469.2</td>
<td>135000.0</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>P-M-EO</td>
<td>2592.5</td>
<td>66.6 ± 5.3</td>
<td>1298.4</td>
<td>33.4 ± 5.3</td>
<td>3890.9</td>
<td>135000.0</td>
<td>2.9 ± 0.2</td>
</tr>
</tbody>
</table>

P-M-C: Meropenem-impregnated beads kept in sterile conditions, but did not undergo sterilization.
P-M-EO: Meropenem-impregnated beads that underwent gas sterilization using ethylene oxide.

Discussion

This in vitro study provides information about meropenem elution after incorporation in a PMMA bead and the feasibility of bead prefabrication and ethylene oxide gas sterilization for future use. Results of our study indicate that meropenem eluted effectively from PMMA and the meropenem concentration on the eluent remained above the breakpoint sensitivity of 4 mcg/ml for 15 days in the P-M-C group and until day 18 in the P-M-EO group. Breakpoint sensitivity limits for other antibiotics commonly incorporated in PMMA beads are as follows: cefazolin, 8 mcg/ml; ciprofloxacin, 1 mcg/ml; clindamycin, 1 mcg/ml; ticarcillin, 64 mcg/ml; tobramycin, 4 mcg/ml; and vancomycin, 5 mcg/ml (22). In a previous study, in vitro elution analysis showed that all the antibiotics maintained concentrations above the breakpoint sensitivity concentrations until day 28, except for ticarcillin and vancomycin (22). In the same study an in vivo elution analysis in sebum fluid revealed that the antibiotic concentrations were similar to the results in vitro (22). Granulation tissue antibiotic concentrations were well above the breakpoint sensitivity concentrations at day 28, except for ticarcillin. Vancomycin and clindamycin were the only antibiotics to attain high concentrations in cortical bone at day 28 (22). Serum antibiotic concentrations were undetectable for most of these antibiotics, except for tobramycin and ticarcillin at three and six hours after bead implantation, but serum values were below toxic concentrations (22).

There was not any zone of growth inhibition observed on any of the samples.
after autoclave sterilization, indicating a complete loss of antibacterial activity. Our study also supports the previous finding that PMMA alone does not have any antibacterial activity under the conditions studied (8, 27–29).

The biphasic elution pattern of meropenem was similar to patterns reported for other antibiotic drugs (8). This biphasic elution is suspected to be the result of initial antibiotic drug elution from the surface of the bead and a sustained release during the second phase of elution from the bead matrix (28). Approximately 40% of the cumulative antibiotic elution occurred during the first hour.

The lack of any significant difference between P-M-EO and P-M-C in the AUC∞ indicated that the total release of meropenem was equivalent. This finding confirms that gas sterilization of meropenem-impregnated PMMA beads with ethylene oxide did not adversely affect antibiotic activity nor elution. Ramos et al reported that neither polymerisation, gas sterilization, nor two-month storage of antibiotic-impregnated PMMA affected the bioactivity of metronidazole and gentamicin against a common pathogen (8). In our study the MRT did differ significantly, suggesting that ethylene oxide sterilization resulted in slightly longer elution time, though there would likely be no clinical significance in this increase (7 hours).

The activity of meropenem is time dependent and the amount of time that an antibiotic concentration is above the MIC is important. In the study reported here, the meropenem concentration in the eluent remained above 4 mcg/ml for 15 days in the P-M-C group and for 18 days if the P-M-EO group. When treating an existing infection, it is recommended that systemic therapy involve the use of effective antibiotic concentrations for three to four weeks or longer depending on response to treatment (7, 30). It is also recommended that a concentration of one to five times the MIC should be maintained for at least half of the dosing interval when time dependent drugs are given systemically (31). However, the concentration that should be maintained over time when a time-dependent antibiotic is used for local treatment is unknown. This should be considered when using meropenem-impregnated beads for treatment of existing infections.

Antibiotic elution rate varies depending on the type of PMMA cement used (27, 28). Simplex™ P radiopaque PMMA cement was selected for this study because it is the most commonly used cement in the United States (5). However, Palacos® bone cement has been found to release the most antibiotic drugs in larger amounts and for longer periods of time than other bone cements due to a larger surface pore size (29, 32, 33). Also, a previous in vitro study evaluating vancomycin elution from PMMA and biodegradable beads reported extended antibiotic elution times when biodegradable beads were used (33).

Implant size also affects antibiotic elution, with smaller beads having a greater surface area and as a result a better elution than larger beads (28). The PMMA beads used in this study were 6.4 mm in diameter as described in previous studies (5, 11). Though biomechanical studies on antibiotic-impregnated PMMA indicate that no more than 10 percent of the total weight should be antibiotic if the cement is used for implant fixation, the percentage can be much higher for bead production. The limit of antibiotic to PMMA ratio recommended for bead production is 1:5. If the amount of antibiotic surpasses this ratio the PMMA will not harden into beads (28).

The volume of PBS used and the elution rates affect the antibiotic concentration in the eluent. The PBS volume chosen for this study was the same as that used in previous studies, although the amount tends to vary among studies (15). The use of serum instead of PBS has not been found to affect elution rates (15).

Beta-lactam antibiotics have been classified as heat-labile antibiotics. The exothermic reaction during polymerisation of PMMA can reach temperatures of 100°C and may result in degradation and inactivation of certain antibiotics (28, 32, 34–38). In a study of meropenem, the MIC for various organisms increased about 16-fold after autoclaving at 121°C for 15 minutes, presumably from alteration of a key portion of the molecule necessary for antimicrobial activity. Autoclave treatment increased the MIC for all the organisms tested, but did not result in complete destruction of the antimicrobial activity. No
change in MIC was observed after heat treatment with 56°C for 30 minutes in a water bath (38). Our study did not support the use of meropenem-impregnated PMMA beads after autoclave sterilization since there was not any zone growth in the P-M-A area, and use of biodegradable materials. The effects and pharmacokinetics of meropenem impregnated PMMA bead implantation should be evaluated in vivo to determine clinical applications.

References