

Research Paper

# Influence of Sonication on Bacterial Regrowth from Antibiotic Loaded PMMA Scaffolds - An *In-vitro* Study

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## Abstract

Periprosthetic joint infection (PJI) is one of the most challenging complications after joint replacement. However, when treated correctly, chances of recovery are good. The most important step in correct diagnosis and management of PJI is the detection of the causative germ. In the last years, the use of sonication in the diagnostic process has become more important. However, this diagnostic methodology has been controversially discussed when used in combination with antibiotic loaded bone cement (PMMA), which is frequently used in joint replacement surgeries. The aim of this study was thus to analyse *in vitro* bacterial growth in sonication fluid cultures obtained from antibiotic loaded PMMA which were contaminated with various bacterial biofilms.

Sonication fluid obtained from antibiotic loaded PMMA (Copal G+V and Copal G+C) and plain Palacos R (control) contaminated either with *S. aureus*, *E. faecalis*, *S. sanguinis* or *P. acnes*, were analysed for bacterial re-growth in a standardised *in-vitro* setting.

*In vitro* bacterial growth was not interfered by released antibiotics from sonication of antibiotic loaded PMMA for *S. aureus*, *E. faecalis* and *S. sanguinis*. However, for *P. acnes* bacterial counts were affected by the released antibiotics as well as by the time delay between sonication and analysis. The *in-vitro* data suggest sonication to be an easy and sensitive diagnostic modality to detect easy-to-detect bacteria, however, results are alarming for the difficult-to-detect bacteria *P. acnes*, indicating that further attention and research is necessary to improve the detection of difficult-to-detect bacteria.

Key words: Periprosthetic joint infection, bone cement, sonication, *in vitro*

## Introduction

Periprosthetic joint infection (PJI) is a major complication after total joint replacement, and leads to additional surgery, causes impairment of function, and produces high costs. There is an increasing incidence of PJI due to an increasing number of operations and patients with an implant *in situ* that have a higher risk of suffering from PJI [1].

The diagnostic and therapeutic management of PJI is demanding and expensive [2]. The accurate detection of the causative bacteria growing in a highly specialized structure known as “biofilm” on the

surface of the implant [3], is one of the most important steps in deciding how a PJI should be treated [4-6]. Tissue samples are regarded as the gold standard in the diagnosis of the causative germ [6, 7]. In the last years various additional methods for biofilm removal like dithiotreitol (DTT) [6] or sonication [4] have been developed and applied in clinical routine. It has been shown that sonication has a high sensitivity and specificity [4] and thus is a widely used method [8, 9].

Bone cement (PMMA) is a well-established material for anchoring artificial joints to the bone [10,

11]. It can be used in combination with antibiotics added to the cement which will be released over a long period of time [12]. It is well known that in vivo, only the antibiotic particles located on the outer surface layer of the cement matrix are released while 90% of the antibiotics added to the cement remain bound to the cement without being released [13, 14]. However, in the literature there is evidence that some of the remaining antibiotics can be released during sonication [15-18]. This could lead to false negative sonication results during the diagnostics of PJI. Other studies however, see no interference in the diagnostics of PJI using sonication and antibiotic loaded PMMA [19]. Thus further clarification on using sonication fluid from sonicating standardized antibiotic loaded PMMA scaffolds, to diagnose PJI, is needed.

Aim of this study was to analyze the effect of sonication of bacterial biofilms attached to standardized antibiotic loaded PMMA scaffolds on in vitro bacterial regrowth.

## Methods

Clinical isolates from a methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus sanguinis* and *Propionibacterium acnes* were obtained from clinical samples causing PJI. Strains were re-grown from cryovial beads and subcultured twice before use. Sensitivity to gentamicin, vancomycin and clindamycin was analyzed by E-test (bioMérieux, Geneva, Switzerland).

## PMMA scaffolds

PMMA cement loaded with 1.25% gentamicin and 5 % vancomycin or 2.50% gentamicin and 2.50% clindamycin, namely Copal G+V and Copal G+C (Heraeus Medical, Wehrheim, Germany) were obtained from the manufacturer as commercially available. Plain Palacos R (without antibiotics, (Heraeus Medical, Wehrheim, Germany) was used as a positive control.

DIN Dynstat™ scaffolds (3x10x15 mm, DIN 53435) were prepared under atmospheric pressure and room temperature (23 °C, humidity 34%) and the given time in the manufacturers' manuals. The doughy cement was then immediately transferred into steel moulds and covered with a plastic shield. The moulds were then transferred into a squeezer. After 20 minutes the hardened cement discs were taken out of the moulds and stored in small plastic containers. Scaffolds were then sterilized by gamma-irradiation and stored at room temperature until use.

## Determination of inhibition zones

All four bacterial strains were suspended in sterile NaCl, the density adjusted to 0.5 McFarland with an optical densitometer (Densimat, bioMérieux, Geneva, Switzerland) and streaked on Columbia agar with 5% sheep blood (bioMérieux, Geneva, Switzerland). Scaffolds were placed on the agar and the plates incubated at 35 °C with ambient air for 16-18 hours (*S. aureus* and *E. faecalis*), at 35°C in 7% CO<sub>2</sub> (*S. sanguinis*) and 36-48 hours at 35 °C anaerobically (*P. acnes*).

## Removal of antibiotics from the scaffolds

Each DIN Dynstat™ scaffold was placed in a 50 mL Falcon tube filled with 10 mL of 1% phosphate buffered saline (Roti®CELL PBS, Carl Roth Laborbedarf, Switzerland), swayed once and stored at room temperature. The PBS was changed once daily for a period of 10-days. For *P. acnes* a 24-days washing sequence was used.

## Biofilm formation

All biofilm experiments were run in sets of 9 scaffolds in parallel. 50 mL Falcon tubes were pre-filled with 9mL Tryptic Soy Broth (TSB, Beton Dickinson AG, Allschwil, Switzerland). Scaffolds were inserted into the Falcon tubes with sterile forceps. Fresh cultures of *S. aureus*, *E. faecalis*, *S. sanguinis* and *P. acnes* were suspended in sterile NaCl and the density adjusted to 0.5 McFarland with an optical densitometer (Densimat, bioMérieux, Geneva, Switzerland). The suspensions were then diluted 1:10 in TSB. In a final step 1000 µl of the diluted bacterial stock solution was added. Scaffolds were then incubated at 35 °C with ambient air (*S. aureus*, *E. faecalis* and *S. sanguis*) for 24 hours or anaerobically (*P. acnes*) for 48 hours without shaking. Negative controls with unwashed scaffolds containing the original amount of antibiotics (Copal G+V and Copal G+C) as well a positive controls without antibiotics in the PMMA (plain Palacos R) were performed in parallel.

## Biofilm analysis

After 24 hours (48 hours for *P. acnes*), the test scaffolds were transferred to new 50 mL Falcon tubes pre-filled with 5 mL PBS with sterile forceps and gently washed 3 times with 1% PBS. Scaffolds were then vortexed for 10 seconds with maximum power, sonicated (BactoSonic, Bandelin, Germany) at 40 kHz for 1 minute and vortexed again for 10 seconds according to an established routinely used clinical procedure [4]. 100 µl sonication fluid was plated after 30, 60, 120, 240 and 480 minutes onto Columbia agar with 5% sheep blood (bioMérieux, Geneva) and incubated at 35 °C with ambient air for 24 h (*S. aureus*

and *E. faecalis*), 24 hours at 35 °C in 7% CO<sub>2</sub> (*S. sanguinis*) and 48 hours at 35 °C anaerobically (*P. acnes*). Bacterial counts were enumerated as >500 CFU/mL, between 100-500 CFU/mL, between 50-100 CFU/mL and <50 CFU/ mL. Additionally, after 480 minutes, 3 mL of sonication fluid was injected in aerobic blood culture bottles for *S.aureus*, *E. faecalis* and *S. sanguinis* (BACTEC Plus Aerobic/F, BD, Allschwil, Switzerland) or anaerobic blood culture bottles for *P. acnes* (BACTEC Plus Anaerobic/F, BD, Allschwil Switzerland) and incubated in the BACTEC FX instrument to check for regrowth [20].

## Results

All tested bacterial strains were vancomycin susceptible according to EUCAST 2015 interpretation. *S. aureus* was also susceptible against gentamicin. *E. faecalis* and *S. sanguinis* showed no high level resistance against gentamicin. For *P. acnes* there are no EUCAST breakpoints for gentamicin (Table 1).

Unwashed scaffolds showed huge inhibition of bacterial growth with *S. sanguinis* and *P. acnes* but small inhibition for *S. aureus* and *E. faecalis*. Scaffolds without antibiotics showed no inhibition in bacterial growth (data not shown). After a 10-days washing period there was no detectable inhibition zone against *E. faecalis*, only a very slight one for *S. aureus* and *S.*

*sanguinis* whereas for *P. acnes* the inhibition zone was still clearly present. After a 17-days washing period a slight inhibition zone against *P. acnes* was visible.

Bacterial growth was not interfered by released antibiotics from the PMMA for *S. aureus*, *E. faecalis* and *S. sanguinis* as all Falcon tubes showed visual turbidity after 24 hours of incubation.

Independent from antibiotic loading, bacterial counts in the sonication fluid were highest (>500 CFU/mL, Figure 1) with *S. aureus* followed by *E. faecalis* and *S. sanguis*. (between 100 and 500 CFU/mL, Figure 2) and remained unchanged for the different time points. Negative controls showed no viable bacteria in the sonication fluid, while positive controls showed the same amount of bacteria in the sonication fluid as the test scaffolds. All 3 strains showed a regrowth in blood culture bottles within 24 hours.

**Table 1:** Minimal inhibitory concentration as determined by E-test

	gentamicin (mg/L)	vancomycin (mg/L)	clindamycin (mg/L)
Methicillin-resistant <i>S. aureus</i>	0.25	0.75	0.064
<i>E. faecalis</i>	12	0.75	8
<i>S. sanguinis</i>	3	0.38	0.094
<i>P. acnes</i>	1	0.19	0.047



**Figure 1:** Bacterial regrowth (methicillin-resistant *S. aureus*, Copal G+V) after 0, 60 and 240 minutes after sonication (representative plates)



**Figure 2:** Bacterial regrowth (*S. sanguinis*, Copal G+C) after 0, 60 and 240 minutes after sonication (representative plates)

For *P. acnes* bacterial counts were always <50 CFU/mL after 0, 60, 120 and 240 minutes and 50% of the scaffolds showed no regrowth after 480 minutes (Figure 3). Positive controls showed a bacterial growth >500 CFU/mL after 0 and 60 minutes which dropped to bacterial counts between 100 and 500 CFU/mL thereafter, while negative controls showed no regrowth. Anaerobic blood cultures bottles remained sterile for 10 days.

## Discussion

PJI can be separated in (i) acute postoperative, (ii) acute hematogenous and (iii) chronic/low-grade infections [21]. While PJI in cases of acute infections usually are caused by virulent bacteria like *S. aureus* or *Streptococcus species*, low-grade infections are more frequently caused by skin flora like *P. acnes* or low virulent germs like *Enterococci* [21].

Accurate diagnostics of the causative bacteria in PJIs is one of the most important steps in the treatment of PJI. In recent years several improvements in the diagnostics like prolonged culture incubation [22], sonication [4, 20], PCR [23] or the use of DTT [24] have been implemented into the clinical routine of PJI diagnosis [8]. Despite all efforts, the rate of culture negative PJI is still around 5-10% [5].

There are various potential reasons for false-negative results. One of the most frequent is antibiotic treatment of the patient 14 days prior to sampling [4]. This is mainly due to a reduced bacterial load in the periprosthetic tissue harvested. Under these circumstances, sonication has been shown to be more accurate than tissue samples in detecting the causative germ, as it is able to dislodge the bacterial biofilm from the surface of the implant [4]. In contrast to the longer antibiotic treatment, the single-shot

antibiotic prophylaxis prior to surgery, seem to influence bacterial sampling less [25].

We aimed to build up a worst-case scenario model for sonication in order to get results relevant for the clinical routine. In clinical routine implants are stored in big plastic containers during surgery, later on implants are covered with liquid for sonication [4]. This results in a big dilution of the antibiotics that could potentially be released. In our experimental setting the volume of the liquid was chosen to result in the least possible dilution, thus resulting in maximal antibiotic interference with bacterial regrowth.

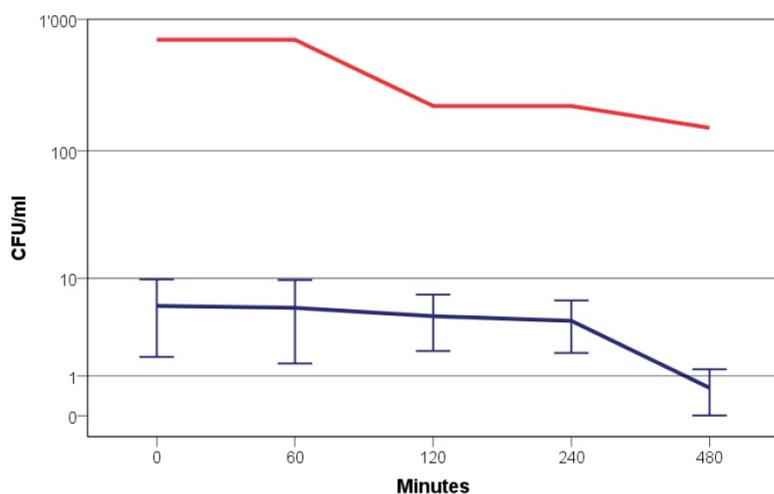
In low-grade infections, bacterial load in periprosthetic tissue is low and culturing might be interfered by problems in processing the scaffolds e.g. anaerobic bacteria like *P. acnes* might no longer grow with delayed transport to the microbiology lab or due to a delay in processing the sonication fluid if loaded with antibiotics. We therefore opted to have several time points for plating the sonication fluid, aiming to analyze the potential effect of released antibiotics on dislodged bacteria, stored in PBS, at different time points.

Bacterial regrowth from *S. aureus*, *S. sanguis* and *E. faecalis* was not influenced by antibiotic release during sonication, as bacterial counts were high (>500 CFU/mL) and comparable to sonication results from positive controls. Furthermore the factor time did not influence bacterial counts, indicating that delayed processing of samples during daily routine has a minor influence.

The results for *P. acnes* are of concern since bacterial regrowth, even immediately after sonication, was low (<50 CFU/mL). No regrowth was detected when plating sonication fluid later than 240 minutes after sonication. In clinical routine all samples would

have been rated negative using a cut-off of >50 CFU/mL [4]. One might argue that biofilm formation on the scaffolds was interfered by released antibiotics. This is rather unlikely since scaffolds placed on agar plates after washing showed no inhibition zone for *P. acnes*.

This study has several limitations: First we only investigated 4 bacterial strains. Choosing the appropriate strains is a crucial step [3]. We choose strains which are often detected to be the causative germ of PJI in clinical daily routine. While *S. aureus* and *E. faecalis* are rather robust, *S. sanguis* and *P. acnes* are more sensitive to growth disturbances. A second weakness of the study, is that *in-vitro* biofilm data is analyzed and



**Figure 3:** Bacterial regrowth on washed scaffolds (*P. acnes*, Copal G+C, blue line, 95% confidence intervals) and positive control (red line).

results are then transferred to an *in-vivo* situation. This application transfer may be difficult to interpret correctly [26]. Thus further studies are needed to see whether our results can be reproduced *in-vivo*. Finally, it remains unclear if potentially more antibiotics could have been released from the PMMA if we would have sonicated the scaffolds longer than 1 minute or with higher energy. However, increasing the sonication time or energy would have destroyed the bacteria wanting to be harvested.

## Conclusion

Sonication did not influence bacterial regrowth from antibiotic loaded PMMA scaffolds for 3 out of 4 bacteria tested. However, the results for *P. acnes*, which are well known to be difficult-to-detect, are of great concern.

## Abbreviations

Periprosthetic joint infection: PJI; dithiotreitol: DTT; Bone cement: PMMA; PBS: Phosphate buffered saline, TSB: Tryptic Soy Broth.

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## Competing Interests

The authors have declared that no competing interest exists.

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