Improved Detection of Biofilm-formative Bacteria by Vortexing and Sonication

A Pilot Study

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Abstract Bacteria such as staphylococci commonly encountered in orthopaedic infections form biofilms and adhere to bone implants and cements. Various methods to disrupt the biofilm and enhance bacterial detection have been reported. We will describe the effectiveness of vortexing and sonication to improve the detection of biofilm-formative bacteria from polymethylmethacrylate by conventional quantitative bacterial culture and real-time quantitative PCR. We used a single biofilm-formative *Staphylococcus aureus* strain and 20 polymethylmethacrylate coupons as an in vitro biofilm model; four coupons were used for each of two control groups or three experimental sonication times (1, 5, and 30 minutes). Vortexing the cement without sonication increased the yield of adherent bacteria to a considerable extent. The combination of vortexing and sonication further enhanced the yield regardless of the duration of sonication. Quantitative

conventional cultures correlated with quantitative PCR assay. The combination of vortexing and sonication to disrupt the bacterial biofilm followed by quantitative PCR and/or culture seems to be a sensitive method for detecting bacteria adherent to bone cement.

Introduction

Prosthetic joint infections are commonly related to bacteria that are protected from antibiotics and the host immune system by a biofilm. The biofilm also makes the bacteria difficult to detect with conventional microbiologic culture. The sensitivity of diagnostic assays has been improved by the use of techniques that dislodge adherent bacteria from the biofilm [13]. Sonication and/or vortexing increases the number of bacteria isolated from retrieved joint implants [8, 14, 16]. Trampuz et al. [14] reported culture of samples obtained by sonication of explanted hip and knee prostheses was approximately 18% more sensitive than conventional culture of periprosthetic tissue among patients undergoing hip or knee revision or resection arthroplasty and was 30% more sensitive in patients who had received antibiotics within 14 days before surgery.

Two-stage revision arthroplasty is recognized as a standard therapy in known infected arthroplasties [1, 2]. During the first operation, the infected material is removed and an antibiotic-containing spacer composed of polymethylmethacrylate (PMMA) is placed. Bacteria adhere to bone cement and form biofilms in vitro [9, 17], even when the cement contains antibiotics. Therefore, it is important to detect biofilm-formative bacteria associated with bone cement. We previously tested optimum conditions to use sonication to dislodge bacteria from stainless steel coupons and determined a relatively short sonication (between 1 and

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5 minutes) was more effective than either shorter or longer periods of sonication to detect biofilm-formative bacteria [4]. However, the optimum conditions to dislodge biofilm-formative bacteria from PMMA have not been investigated.

Molecular methods, especially PCR, have been recognized as a sensitive tool for improving the diagnosis of prosthetic joint infections [5, 15]. Mariani et al. [7] suggested PCR may be more sensitive than conventional cultures and some cases of prosthetic joint infection may be misclassified as aseptic loosening using current culture methods.

We hypothesized dislodging biofilm-formative bacteria from PMMA by vortexing and/or sonication may improve bacterial identification sensitivity. More specifically, this study was performed to answer the following questions: (1) Do vortexing and sonication increase the number of biofilm-formative *Staphylococcus aureus* isolated from PMMA by conventional quantitative culture and real-time quantitative PCR (qPCR)? (2) Does the length of sonication time influence the number of dislodged bacteria? (3) Does qPCR correlate with the results of conventional quantitative culture as a method to detect bacteria?

Materials and Methods

We used biofilm-formative *Staphylococcus aureus* (ATCC 12600) for this in vitro study. Twenty PMMA coupons (Stryker Orthopaedics, Mahwah, NJ) were prepared without antibiotics; the coupons (diameter, 26 mm; thickness, 3 mm) contained 9.1% barium sulfate. We used four coupons for each of two control and three experimental groups. The two control groups included coupons that were neither vortexed nor sonicated (V– S– group) and coupons that were vortexed but not sonicated (V+ S– group). The three experimental groups (V+ S+ groups) were all vortexed and sonicated for three different times: 1, 5, and 30 minutes (V+ S1 min, V+ S5 min, and V+ S30 min groups, respectively).

Each coupon was placed in 10 mL tryptic soy broth (TSB) medium containing 1.5×10^4 colony-forming units (CFU)/mL of biofilm-formative *Staphylococcus aureus* bacteria and incubated initially for 4 hours without shaking. The coupons were washed once with 25 mL phosphate-buffered saline (PBS), transferred to a sterile container with fresh TSB, and reincubated for 15 hours to allow biofilm formation. The coupons then were washed twice with 25 mL PBS to remove any nonadherent bacteria from the surface and transferred to a new sterile cup with 25 mL PBS. The coupons were vortexed for 30 seconds inside the cup (Maxi Mix[®] II; Barnstead International, Dubuque, IA) and then subjected to sonication at a frequency of

40 kHz (Branson Ultrasonic Cleaner; Branson Ultrasonics, Danbury, CT) for different times, followed by additional vortexing for 30 seconds.

For quantification of bacteria by conventional microbiologic culture, an aliquot of the suspension was serially diluted and spread in duplicate on blood agar plates (Trypticase[™] Soy Agar with 5% Sheep Blood; Becton, Dickinson and Co, Sparks, MD). After incubation for 18 to 24 hours, we counted CFU for each plate, averaged for each group, and expressed the results as CFU/mL. For quantification by qPCR targeting 16S rDNA and *tufA* gene, 1 mL of each sonicate solution was heated at 100°C for 10 minutes, and DNA extraction was performed using Easy-MAG[™] (Biomerieux, L'Etoile, France) according to the manufacturer's instructions. Twenty-five microliters of DNA extract was obtained for each sample. qPCR assays targeted 16S rDNA [5] and *tufA* DNA using a Rotorgene 3000[®] (Corbett Research, Sydney, Australia). The *tufA* primer sequences (BioChem, Salt Lake City, UT) were 5'-ATGCCACAACCTCGTGA-3' (forward primer) and 5'-ACCACGACCAGTGATTGAGAA-3' (reverse primer).

The PCR mixture consisted of 4.0 or 4.5 mmol/L MgCl₂, 0.2 μmol/L of each primer, 12.5 μL 2x Sensi-Mix[™] (Quantace, Norwood, MA), and 0.5 μL 50x SYBR[®] Green I (Quantace) for a volume of 23 μL master mix. Two microliters of DNA extract was added to the reaction mixture for a final reaction volume of 25 μL for each tube. The cycling conditions for 16S rRNA were –95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 57°C for 20 seconds, and 72°C for 15 seconds; the cycling conditions for *tufA* were –95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 50°C for 30 seconds, and 72°C for 15 seconds. The number of bacteria was derived from a standard curve established previously and included in each PCR run.

One-way analysis of variance was used to compare culture and qPCR means across the five groups (two control and three experimental groups). Differences between the five groups were compared using Student-Newman-Keuls test. The test was chosen to accommodate the large number of comparisons relative to the size of the data. Associations between the culture and qPCR results were evaluated using Pearson's correlation coefficient.

Results

The use of vortexing without sonication increased ($p = 0.025$) the sensitivity of the microbiologic culture when compared with culture without vortexing. Sonication in combination with vortexing further enhanced (V– S– versus V+ S1 min and V+ S5 min groups, $p = 0.015$;

V- S- versus V+ S30 min groups, $p = 0.004$) the detection of bacteria when compared with culture alone; however, there was no difference between the V+ S- group and the three V+ S+ groups (Fig. 1A). All 20 samples were positive by PCR. qPCR results targeting *tufA* were similar to those of culture (Fig. 1B), ie, vortexing samples yielded more bacteria than not vortexing (V- S- versus V+ S- groups, $p = 0.025$; V- S- versus V+ S+ groups, $p = 0.001$). In addition, the results of the 16S rDNA assay showed increased detection when samples were sonicated in addition to being vortexed (V+ S- versus V+ S1 min groups, $p = 0.013$; V+ S- versus V+ S5 min and V+ S30 min groups, $p < 0.001$). The difference between the different assays run on the same samples is most likely the result of the higher variability in

the results of the V+ S- group using conventional culture and *tufA* DNA PCR (Fig. 1A–B).

The duration of sonication did not influence the microbiologic sensitivity. More specifically, the numbers of cultivated bacteria (CFU/mL) were similar among the 1-, 5-, and 30-minute sonication groups (Fig. 1A). Similarly, the duration of sonication did not influence the number of bacteria identified using either the 16S rDNA or *tufA* PCR assays (Fig. 1B–C).

There was good correlation between culture and 16S rDNA PCR results ($r = 0.776$; $p < 0.001$) (Fig. 2A) and between culture and *tufA* gene PCR results ($r = 0.741$; $p < 0.001$) (Fig. 2B). The standard curve run with each PCR assay showed good reproducibility as to Ct (cycle threshold) values.

Fig. 1A–C (A) Results of quantitative culture of PMMA coupons under biofilm-formative conditions are shown as a function of different treatments. We found fewer bacteria in the V- S- group compared with the V+ S- group and the three V+ S+ groups. The numbers of cultivated bacteria (CFU/mL) were similar among the V+ S- group and the 1-, 5-, and 30-minute V+ S+ groups. (B) *tufA* qPCR results by sonication time with vortexing in biofilm-formative conditions are shown. Vortexing samples yielded more bacteria than

not vortexing. Bacterial numbers were similar among sonication groups regardless of the duration of sonication in *tufA* PCR assays. (C) 16S rDNA qPCR results by sonication time with vortexing in biofilm-formative conditions are shown. Results of the 16S rDNA assay showed increased detection when samples were sonicated in addition to being vortexed. Bacterial numbers were similar among sonication groups regardless of the duration of sonication in 16S rDNA assays. The exact probability values are shown.

Fig. 2A–B (A) We observed a correlation between culture and 16S rDNA PCR assay in biofilm-formative conditions and (B) between culture and *tufA* gene PCR assay in biofilm-formative conditions.

Discussion

Dislodging biofilm-formative bacteria from orthopaedic devices or other implanted materials by vortexing and sonication may identify bacteria in a situation thought to be aseptic. This study focused on attempting to improve the way in which microbiologic cultures and PCR are used to detect biofilm-formative bacteria adherent to PMMA bone cement commonly retrieved at revision arthroplasty or in the second-stage reconstruction of a previously infected arthroplasty. We posed the following questions: (1) Do vortexing and sonication increase the number of biofilm-formative *Staphylococcus aureus* isolated from PMMA by conventional quantitative culture and qPCR? (2) Does the sonication time influence the number of the dislodged bacteria? (3) Do the results of qPCR correlate with the results of conventional quantitative culture as methods to detect the bacteria?

Our study has several limitations. First, we did not include a sonication-only group in our experiments. Therefore, we could not tell how effective sonication alone might be to dislodge biofilm-formative bacteria compared with vortexing alone or the combination of both. Second, we evaluated only one strain of one bacterial species (*Staphylococcus aureus*) on one material (PMMA) at one incubation duration (15 hours). van de Belt et al. [17, 18] reported on biofilm formation of *Staphylococcus aureus* on six different bone cements loaded with or without gentamicin and suggested biofilm formation on bone cements may be dependent on properties of the cement surface such as its roughness and porosity. Compared with our in vitro results, bone cements retrieved from clinical patients under various circumstances, including different kinds of cements, different bacteria, different durations of infections, and variability in antibiotic load and antibiotic susceptibility of the bacteria, may bring different consequences.

Numerous investigators who have focused on biofilm have used various methods to dislodge the bacteria adherent to biomaterials surfaces. For example, Oga et al. [9, 10] used a rinse method with saline, whereas Olson et al. [11] used vortexing for 1 minute without sonication. Moreover, sonication with or without vortexing has been

used widely [14, 16]. The differences in methods to dislodge biofilms make direct comparisons difficult. Padberg et al. [12] evaluated methods of enhancing bacterial recovery from Dacron[®] graft cylinders (vascular prostheses) seeded with commonly encountered bacterial pathogens by quantitative culture techniques. They compared three methods: (1) ultrasonic bath treatment; (2) direct ultrasonic disruption; and (3) agitation on a vortex mixer in vitro in broth medium. They reported ultrasonic bath treatment was superior to vortexing and direct ultrasonic disruption. Our results of conventional culture and qPCR, however, suggest vortexing substantially improved detection of bacteria adherent to bone cement and the combination of vortexing and sonication yielded optimum results.

Although the duration of sonication is variable between different institutions, sonication times between 1 minute and 30 minutes are commonly used [8, 14, 17]. In our previous study [4], the most bacteria adherent to stainless steel plates were recovered at 1 minute of sonication, whereas prolonged sonication was inferior. The duration of the sonication time tested in the current study did not make any difference in detection of bacteria. This may be because a sonication time of 1 minute was already sufficient to dislodge all bacteria attached to the PMMA coupons in our in vitro study with a relatively short biofilm formation time. Additionally, our data suggest ultrasound for 30 minutes did not influence bacterial viability, although it has been reported long durations of sonication damage bacterial viability [4]. The discrepancies between this study and our previous study [4] may be related to differences in composition and surface properties between PMMA and a metal plate substrate.

We found a correlation between conventional culture and PCR results. Like conventional culture, PCR may be useful not only for identifying bacteria [6] but also for quantifying bacterial concentration, although care must be taken because PCR may not distinguish live from dead bacteria [3].

Vortexing is a convenient, inexpensive, widely available technique and is effective in dislodging bacteria adherent to bone cement. Because we observed no differences among 1, 5, and 30 minutes of sonication, a sonication time of approximately 1 minute may be practical for clinical use.

The techniques of vortexing with or without sonication could be relatively easily introduced into the routine microbiologic laboratory. Although care must be taken when interpreting the current information for clinical use, these simple methods may help improve detection of persistent infection in patients undergoing the second stage of rerevision arthroplasty for a known infection. The methods also may be used to help recognize infection in patients believed to have experienced aseptic loosening of cemented total hip and knee implants.

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