# Variability of Sonication to Dislodge *Staphylococcus aureus* Biofilm from Titanium Discs: An *In Vitro* Study

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INTRODUCTION: Implant-associated infections require an accurate diagnosis; however, bacterial biofilms on implant surfaces hamper correct microbial diagnosis [1]. Sonication has been found to be a practical and effective way to physically remove biofilm from implant surfaces [2]. Unfortunately, the amount of biofilm being dislodged from the surface of the implant has also been reported to be variable using 5-minute sonication [3]. It is important to find an effective biofilm dislodgment method without killing the viable bacteria removed. In the present *in vitro* study, we have compared the efficacy of three sonication protocols for dislodging biofilm from orthopaedic relevant implant surfaces, to compare the differences in sonicated bacterial activity and the remaining biofilm structure before and after sonication.

METHODS: *Staphylococcus aureus* ATCC 25923 biofilms were grown on corundum blasted Ti6Al4V discs for 2 days (less mature) or 7 days (more mature). The bacteria ($10^7$ CFU/ml) were initially adhered for 4 hours at 37°C on the titanium discs and rinsed with phosphate buffer saline (PBS). Followed by immersion in 2 ml tryptic soy broth, with media refreshment every 2 or 3 days. After the growth period, the titanium discs were rinsed with PBS to remove any planktonic bacteria. The biofilms in 5 ml PBS were detached using three different sonication methods (5 minutes, 10 minutes or 1-minute vortex followed by 10-minute sonication). The effect of the sonication was quantified by counting colony forming units (CFUs) from the sonication fluid (n=6). A qualitative analysis of the biofilm on the titanium discs before and after sonication was also performed by scanning electron microscopy (n=4).

RESULTS SECTION: As expected, 7-day biofilms showed a 1-log increase in CFU count compared to 2-day biofilms, although only significant for the 10-minute sonication method (student t-test). The 5-minute sonication method had the highest CFU count compared to 10-minute sonication or 1-minute vortex followed by 10-minute sonication (Figure 1). SEM images revealed biofilm formation for both 2-day and 7-day biofilm, where 7-day biofilm showed more extracellular polymeric substances (EPS) (Figure 2). The 5-minute sonication was variable at removing all of the biofilm and clumps of bacteria were still visible in certain areas and 10 minutes sonication to a lesser extent of variability, with more unremoved bacterial areas seen on day 7. One-minute vortex combined with 10-minute sonication was the best sonication method to remove the biofilm consistently.

DISCUSSION: Our results show that there is variability using 5-minute or 10-minute sonication alone in mechanically removing *S. aureus* biofilm from titanium discs. The variability in biofilm removal was more pronounced for 7-day biofilm probably due to the biofilm attaching more tightly to the titanium as EPS provides structural stability. The 1-minute vortex combined with 10-minute sonication effectively removed the biofilm from the surface for both 2-day and 7-day biofilm, albeit decreasing the CFU count compared to 5-minute sonication alone; however, this was not significant. These results show the importance of the sonication method employed when studying biofilm on titanium implants, as there may be remaining biofilm on the surface resulting in an underestimation of technology efficacy. This could also have an effect in the clinic, as some bacterial species may not be identified in the infection, due to variable biofilm removal caused by an inadequate sonication technique employed.

SIGNIFICANCE/CLINICAL RELEVANCE: Infection of porous titanium implants is a major clinical issue; hence it is vital to study the biofilm formed on these titanium surfaces. This study shows the importance of including vortexing prior to sonication to remove consistently bacterial biofilm from titanium discs and to standardize methodologies.

REFERENCES:

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IMAGES AND TABLES:

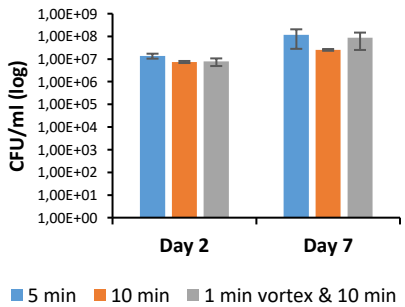


**Figure 1. Colony forming units/ml (± standard error) in a *S. aureus* biofilm culture after different sonication methods.** Biofilms were grown on orthopaedic relevant titanium discs for 2 days or 7 days. Thereafter, the biofilm was removed using water bath sonication either 5 minutes, 10 minutes or 1 minute vortex and 10 minutes sonication (n=6).

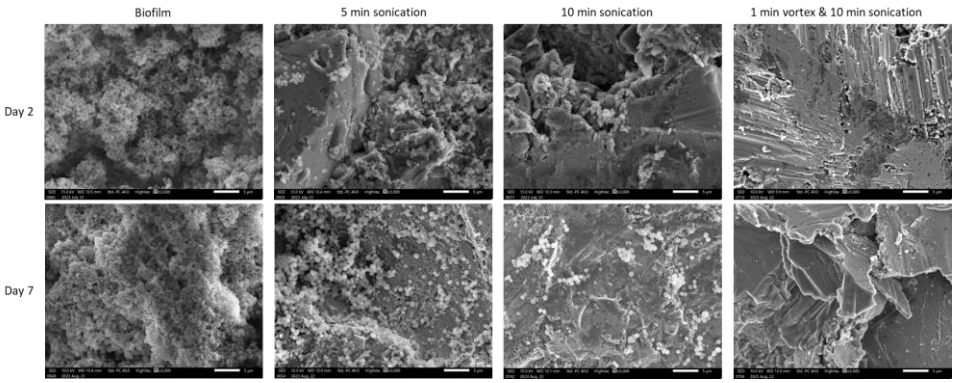


**Figure 2. Scanning electron microscopy images of 2-day and 7-day *in vitro S. aureus* biofilms on titanium discs before and after various sonication biofilm removal methods.** Representative images (n=4) were taken at 3000X magnification. Scale bar = 5 µm.