



# Effects of Low-Frequency Ultrasound Applied *In Vitro* to Highly Antibiotic Resistant *Acinetobacter* Isolates Recovered from Soldiers Returning from Iraq

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## Abstract

Brooke Army Medical Center isolated 25 highly antibiotic resistant *Acinetobacter* ssp. (primarily *A. baumannii*) from wounded soldiers returning from Iraq. Concern about effective treatment of these organisms led our institution to begin investigating low frequency ultrasound (LFU) as a method of increasing the effectiveness of antibiotics on *A.baumannii* in wound management.

Studies have suggested that LFU applied in conjunction with antibiotics may increase their overall effectiveness. Additional studies have shown that LFU has the potential to be an effective wound debridement tool. We hypothesize that combining antibiotics with LFU may be an effective method of wound management and that this combination may be synergistic in its overall effect. In this initial work, we wanted to determine if sonication would have an effect on our organism of interest, *A. baumannii*. We selected several organisms, both gram positive and gram negative, that have been shown to be killed by sonication (*E. coli*, *S. aureus*, and *S. pyogenes*) and added three highly resistant *A. baumannii* isolates.

We used a previously described protocol for the basic set up of our *in-vitro* model. LFS was achieved using the SONOCA 180 from NAS/CORP-Telmah. The SONOCA 180 is designed with a fluid feed to transmit the sound waves and to prevent overheating. There was no need to use the fluid feed to transmit the sound waves because our bacterial suspension was already in liquid. We held the tubes in an ice bath to prevent overheating. Temperature was controlled, as measured temperatures ranged from 21-27° C.

Prior to sonication, the bacterial suspension was set to a 0.5 McFarland (MF) standard and then serially diluted to approximately 100,000 CFU/mL. Initial colony counts were taken prior to sonication. We then sonicated at 60% output in ten second bursts, followed by 50 second cool down periods, until a total of 120 seconds of sonication was achieved. Aliquots were taken and plated after each 20 seconds of sonication. Bacterial death was measured by colony counts after 24 hours of CO<sub>2</sub> enhanced incubation. In addition, we made visual observations using standard protocols for Acridine Orange and BacLight stains. The BacLight may be useful for future counts utilizing flow cytometry.

Our data for *S. aureus*, *S. pyogenes*, and *E. coli* were similar to those of previous experiments (figure 3). Colony counts were significantly reduced by sonication (figure 4 and 5). Furthermore, *A. baumannii* colony counts were also greatly reduced by sonication (figure 6). Actual cell destruction was also visualized using acridine orange and BacLight staining (figures 7-10). Our data supports the assertion that sonication has an antibacterial effect on some bacteria, including *A. baumannii*. Our next step is to add antimicrobial agents and determine if their effectiveness can be increased by sonication.

## Materials and Methods

We approximated a 1.0X10<sup>6</sup> colony forming units per milliliter (CFU/ml) suspension of each of the organisms studied by taking a 0.5 MF solution (1.5X 10<sup>8</sup> CFU/ml) and serially diluting with 0.45% saline until the expected concentrations were achieved. Aliquots were taken from the each suspension and served as the baseline viability (0% sonication). During sonication the suspension was placed into an ice bath to control temperature. We used the SONOCA 180 with a three ball probe and exposed each bacterial suspension to alternating 10 second cycles of LFU followed by 50 seconds of rest (to prevent overheating) until the suspensions received a total of 120 seconds of sonication. Aliquots of the bacterial suspension were removed after each 20 seconds of exposure and then inoculated onto blood agar plates and incubated for 24 hours at 37°C in a CO<sub>2</sub> enriched environment. CFU's were then enumerated and the results from three separate experiments were averaged. Each aliquot was stained using both BacLight and Acridine Orange using standard protocols.



Figure 1  
This is the basic set-up for our model. The conical tube was put into an ice bath to prevent overheating.



Figure 2  
The SONOCA 180 was used to produce the sonication. Fluid normally flows through the tip to give the sonic waves a medium through which to travel. It also provides cooling for the tip.

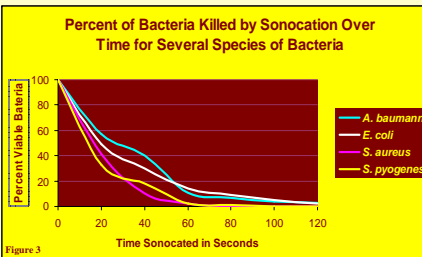


Figure 3

Each organism had a similar decline in viable cell count. The gram positive organisms approached 0% viable colony forming units (CFU) at 60 seconds. The Gram negative organisms showed a more gradual decline for the entire 120 seconds of sonication, finishing at 2-4% of the original viable CFU count.



Figure 4 and 5 show the difference between an untreated plate and a plate exposed to 40 seconds of sonication in one of our preliminary tests.

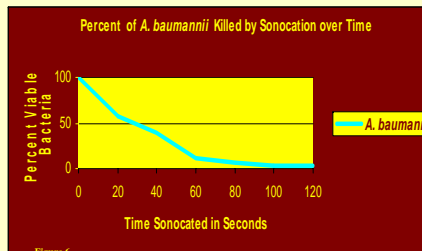


Figure 6

This graph shows that the *A. baumannii* follows a similar decrease of CFU

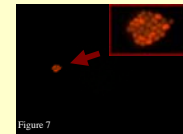


Figure 7  
This is an unsonicated *A. baumannii* isolate stained with Acridine Orange.

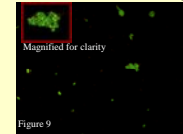


Figure 9  
This is an unsonicated *A. baumannii* isolate stained with BacLight stain.



Figure 8  
After two minutes of sonication the isolate from figure 7 is reduced to debris.

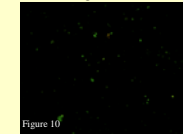


Figure 10  
Once again, the isolate from figure 9 is visually destroyed by sonication.

## Discussion and Conclusion

Our results suggest that sonication does have a bactericidal effect. Our next question is whether adding an antimicrobial agent to sonication would act in a synergistic fashion and allow for greater killing with decreased sonication. We tried several stains to visualize cell death, however the sonication obliterated cells, making it difficult to determine a ratio of living to dead cells visually. We are looking at alternate counting methods, including using DNA intercalation dyes and flow cytometry.

We minimized the effect of heat generated by the low-frequency ultrasound by using an ice bath and short bursts of sonication followed by longer rest periods. This kept the temperature from rising to levels which could potentially kill cells. Temperature changes were slight, with the greatest change being 6° C. The ice bath is unnecessary *in vivo* because the constant water supply that is supplied cools the area.

Due to the potential for aerosolization, all work was performed using proper precautions. We chose this model as first step in measuring the effectiveness of sonication on *Acinetobacter baumannii* because it was previously shown to be successful on other organisms. These initial results show sonication has a bacteriocidal effect at the concentrations tested and suggest that this may be beneficial in early wound treatment.

Other researchers have shown that sonication is effective in the debridement of diabetic wounds. A logical next step would be to use the SONOCA 180 in an animal wound model to measure its effectiveness in cleaning recent wounds. This would help determine the utility of sonication in the field hospital for early wound management.