Short communication

Ultrasound affects minimal inhibitory concentration of ampicillin against methicillin resistant Staphylococcus aureus USA300

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Running Title: Ultrasound effect on MRSA susceptibility to ampicillin

SUMMARY

Antimicrobial resistance is one of the most serious global public health problems. Therefore, novel strategies are needed to counteract bacterial resistance development. The aim of the present study was to enhance the activity of antibiotics to bacteria by using ultrasound. Ultrasound reduced the dosage of ampicillin required to impair bacterial viability.

Key words: MRSA; ultrasound; ampicillin; antimicrobial; sonowell

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The widespread use of antibiotics has resulted in a growing problem of antimicrobial resistance in community and hospital settings (Mai et al., 2017). Antimicrobial classes for which resistance has become a worrisome problem include β-lactams, glycopeptides, and fluoroquinolones (Rice, 2012). Therefore, novel antimicrobial drugs are needed to counteract bacterial resistance development (Klahn et al., 2017). However, the increasing outbreak of various multidrug-resistant (MDR) *Staphylococcus aureus* strains has resulted in treatment difficulties, which imposes a burden on health-care systems and intensifies the need for new antimicrobial agents (Dudhagara et al., 2014).

Methicillin-resistant *S. aureus* (MRSA) causes numerous infections ranging from mild cellulitis to life-threatening sepsis. (Stryjewski et al., 2014; Mlynek et al., 2016). Furthermore *S. aureus* can invade eukaryotic cells and so evade antimicrobial therapy (Artini et al., 2011; Papa et al., 2013; Selan et al., 2017).

Although many MRSA infections are health-care associated, the increasing incidence of community-acquired MRSA (CA-MRSA) infections represents a growing public health concern (Otto, 2013). CA-MRSA USA300 is responsible for many kinds of infections of skin and soft-tissue.

**Previous experiments reported in literature have shown a synergistic effect between ultrasound and antibiotics in killing bacteria belonging to Gram positive and Gram negative (Rediske et al., 35 1998; Carmen et al., 2004).**

A recent review addresses that the effect of ultrasound on reduction of MIC depends both on bacterial species and on antibiotic class (Cai Y et al., 2017) rather than Gram positive and negative distinction.

In the present work we used a novel instrument (SonoWell®, Promedica Bioelectronics srl, Italy) designed specifically to work on cell cultures grown in plates. The instrument is equipped with 4 flat ultrasound transducers operating respectively at 0.65, 1.0, 2.4 and 4.5 MHz driven by 4 parallel channels each able to generate and amplify its respective ultrasound frequency. Suitable protocols varying the spatial peak temporal average intensity (ISPTA) from 0.2 mW/cm² up to 12 W/cm² for each channel were devised to deliver similar acoustic pressures at the different operating frequencies. Peak pressures (in kPa) delivered vs ISPTA within the well were measured with hydrophones (Precision Acoustics, UK) to establish reference calibration curves.

**The instrument described in this manuscript represents a novel apparatus where the frequencies, the acoustic pressure and the time of exposure are completely different from data reported in literature.**

To assess the influence of ultrasound on the bacterial tolerance to antibiotics, we adopted a condition of sonication that doesn't influence bacterial viability. A residual vitality of at least 90% of bacterial cells was the criterion adopted to find the cut-off value for maximal sonication intensity.

The bacterial strain used was MRSA USA300 (Papa et al., 2013; Kaïret et al., 2017). Overnight bacterial culture of USA 300 grown in Brain Heart Infusion (BHI) broth at 37 °C under vigorous
agitation (180 rpm) was diluted 1:100 in 24-wells and subsequently treated. The sonication conditions are shown in Table 1.

Sonications were performed with all four transducers, each one was used at three different acoustic intensities (I_{SPTA}), resulting in three different acoustic pressures (Table 1). Each well was sonicated for 180 seconds with an ON/OFF time of 15/5 ms, Duty Cycle 75%. **Furthermore, the cavitation effect for the experimental conditions here adopted is neglectionable.**

Figure 1 shows the spatial distribution in the multi-well plate of the experimental conditions described above. To avoid interference between adjacent wells, the non-sonicated controls (NT) were centrally placed (Figure 1).

Each well was inoculated with a bacterial suspension of \(10^5\) in a total volume of 1 mL. The plate was kept at 37 °C throughout the experimental phase. After sonication the content of each well was recovered and appropriate dilutions were seeded on an agar plate. After overnight incubation at 37°C, the plates were retrieved and the colony forming units (CFUs) were measured.

The results obtained demonstrated that there are no interference effects between adjacent wells. The adopted experimental conditions were compatible with bacterial viability. However, in the extreme condition (acoustic pressure 500 kPa - 4.5 MHz transducer) a not statistically significant 1-log reduction of bacterial load was observed.

On the basis of the results obtained in preliminary experiments, we used 4.5 MHz transducer at acoustic pressure of 250 kPa. Only this transducer induced a reduction of bacterial viability after acoustic pressure increase from 250 to 500 kPa. Then the defined sonication conditions were tested on the minimal inhibitory concentration (MIC) of ampicillin on MRSA USA 300. Ampicillin has been chosen for different reasons: i) MRSA USA300 is normally resistant to it; ii) it irreversibly impairs the cell wall synthesis; iii) it is a broad-spectrum antibiotic.

Logarithmic-phase culture of *S. aureus* USA300 was added to each well to achieve \(10^6\) CFU/tube. The multiwell was incubated at 37 °C for 18 h. After incubation the MIC was recorded as the lowest concentration of antibiotic that completely inhibits visible growth of the organism (according to NCCLS). Ampicillin MIC value for MRSA USA 300 is 10 mg/mL.

The experiment was carried out using scalar dilutions of ampicillin starting from 10 mg/mL. Wells were sonicated as described above. To amplify the sonication effect, the treatment was repeated three times on the same well at intervals of 15 minutes.

Immediately, after sonication, the content of each well was recovered and appropriate dilutions were seeded on agar plate. After overnight incubation at 37 °C, CFU counts on the plates were measured.
Each data point was obtained from six independent experiments, each performed at least as quadruplicates. Results obtained showed a reduction of MIC value from 10 mg/mL to 5 mg/mL in three out of six performed experiments. In order to improve the activity of ampicillin on the cells after sonication, we used the 4.5 MHz transducer with an acoustic pressure of 500 kPa. Six independent experiments were performed in quadruplicates also in this case. Data obtained showed a reduction of MIC value in six experiments out of six, and specifically to 2.5 mg/mL (four experiments) and to 5 mg/mL (two experiments), respectively. The differences obtained in MIC values for 4,5 MHz transducer at the various power levels are statistically significant ($p$ value < 0.05). Results are summarized in Figure 2.

Results obtained demonstrated that US is able to reduce the effective dosage of ampicillin to impair the bacterial viability. This effect is correlated to the frequencies of US used, though in this field of application, further studies are needed.

Furthermore, it is important to underline that the intensity of ultrasounds used in these experiments are absolutely safe for human tissue. The British Medical Ultrasound Society published the guidelines for the safe use of diagnostic ultrasound equipment. Ultrasound is considered safe up to a mechanical index (MI) value < 0.7. In our equipment, using the 4.5 MHz transducer at 500 kPa, the MI is 0.23, notably lower than the MI reported in the guidelines.

In order to obtain an effective improvement of the bacterial response to antibiotic is important to find a right combination between acoustic pressure and frequency because a high power does not necessarily correspond to a better microbiological effect.

Application of ultrasound in combination with antibiotic therapy could therefore lead to better treatment of bacterial infections.

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Competing interests
The authors have no competing interests to declare.
References


Table 1: Summary of sonication conditions.

<table>
<thead>
<tr>
<th>Transducer</th>
<th>Acoustic Pressure Peak (kPa)</th>
<th>Acoustic Intensity $I_{SPTA}$ (W/cm^2)</th>
<th>WELL-TIME (sec)</th>
<th>DUTY CYCLE</th>
<th>DEAD TIME (sec)</th>
</tr>
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<tbody>
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<td>0,65 MHz</td>
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<td>180</td>
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<td>10</td>
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<tr>
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<td>180</td>
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<tr>
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<td>180</td>
<td>75%</td>
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</tbody>
</table>
Figure 1
Spatial distribution of the different experimental conditions in the 24-well plate. Each well was seeded with 1mL of bacterial culture (10^5/mL). NT indicates not sonicated controls.
Figure 2
Effect of ultrasounds on ampicillin MIC of MRSA S. aureus USA300. Bars correspond to weighted averages, error bars represent standard deviations.