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

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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.

In the present work we used a novel instrument (SonoWell®, Promedica Bioelectronics srl, Italy) designed specifically to work on cell cultures grown on 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 paper is a novel apparatus in which frequencies, acoustic pressure and time of exposure are completely different from data reported in the literature. To assess the influence of ultrasound on bacterial tolerance to antibiotics, we adopted a condition of sonication that doesn't influence bacterial viability. 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.

Sonifications were performed with all four transducers, each of which was used at three different acoustic intensities (Iₚₑ, tₛ), 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%. The cavitation effect for the experimental conditions adopted here is neglectable. Figure 1 shows the spatial distribution in the multi-well plate of the experimental conditions described above. To avoid interference between adjacent wells, 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 statistically non-significant 1-log reduction of bacterial load was observed.

Based on the results obtained in preliminary experiments, we used a 4.5 MHz transducer at an acoustic pressure of 250 kPa. Only this transducer induced a reduction of bacterial viability after acoustic pressure increased from 250 to 500 kPa.

The defined sonication conditions were then tested on the minimal inhibitory concentration (MIC) of ampicillin on MRSA USA 300. Ampicillin was chosen for a number of reasons:

1) MRSA USA300 is normally resistant to it;
2) irreversibly impairs cell wall synthesis;
3) 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 hours. 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.

### Table 1 - Schematic representation of sonication conditions.

<table>
<thead>
<tr>
<th>Transducer</th>
<th>Acoustic Pressure Peak (kPa)</th>
<th>Acoustic Intensity ISPTA (W/cm²)</th>
<th>WELL-TIME (sec)</th>
<th>DUTY CYCLE</th>
<th>DEAD TIME (sec)</th>
</tr>
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<tbody>
<tr>
<td>0.65 MHz</td>
<td>30</td>
<td>0.29</td>
<td>180</td>
<td>75%</td>
<td>10</td>
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<tr>
<td>0.65 MHz</td>
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<td>120</td>
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<tr>
<td>1 MHz</td>
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<td>0.29</td>
<td>180</td>
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<tr>
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<td>500</td>
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<tr>
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</tr>
<tr>
<td>4.5 MHz</td>
<td>500</td>
<td>5.20</td>
<td>180</td>
<td>75%</td>
<td>120</td>
</tr>
</tbody>
</table>

Figure 1 - Schematization of spatial distribution in the 24-well plate of the experimental conditions used. Each well was seeded with 1 mL of \(10^5\) bacterial cultures. NT represents the not sonicated controls.

Figure 2 - Effect of ultrasounds on ampicillin MIC on MRSA \(S.\) aureus USA300. Data are reported as weighted average. Error bars represented standard deviations.
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. The content of each well was recovered immediately after sonication 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 experiments. To improve the activity of ampicillin on cells after sonication, we used the 4.5 MHz transducer with an acoustic pressure of 500 kPa. Six independent experiments were performed in quadruplicates here as well. Data obtained showed a reduction of MIC value in all six experiments, specifically to 2.5 mg/mL (four experiments) and to 5 mg/mL (two experiments). The differences obtained in MIC values for the 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 bacterial viability. This effect is correlated to the frequencies of US used, although further studies are needed in this field of application. It is important to specify that the ultrasound intensities 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. It is important to find the right combination of acoustic pressure and frequency to achieve an effective improvement of bacterial response to an antibiotic, because high power does not necessarily correspond to better microbiological effect. The 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