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Nanomotion Detection Method for Testing Antibiotic Resistance and Susceptibility of Slow-Growing Bacteria

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Infectious diseases are caused by pathogenic microorganisms and are often severe. Time to fully characterize an infectious agent after sampling and to find the right antibiotic and dose are important factors in the overall success of a patient's treatment. Previous results suggest that a nanomotion detection method could be a convenient tool for reducing antibiotic sensitivity characterization time to several hours. Here, the application of the method for slow-growing bacteria is demonstrated, taking *Bordetella pertussis* strains as a model. A low-cost nanomotion device is able to characterize *B. pertussis* sensitivity against specific antibiotics within several hours, instead of days, as it is still the case with conventional growth-based techniques. It can discriminate between resistant and susceptible *B. pertussis* strains, based on the changes of the sensor's signal before and after the antibiotic addition. Furthermore, minimum inhibitory and bactericidal concentrations of clinically applied antibiotics are compared using both techniques and the suggested similarity is discussed.

1. Introduction

Infectious diseases are one of the most important causes of human mortality worldwide. Therefore, rapid detection and identification of microbial pathogens are mandatory in order to treat patients appropriately. It is important to realize that antimicrobial susceptibility testing (AST) should be carried out for all pathogens recovered from an infectious process, since therapeutic measures cannot be consistently predicted based exclusively on the knowledge of the identity of the infecting microorganisms. Consequently, it is evident that physicians require rapid and reliable AST that allows them to make a fast decision and, as a result, improve patient outcomes and reduce healthcare-associated costs. A number of laboratory methods are currently available to characterize the in vitro susceptibility of microorganisms against antibiotics. Current culture-based methods include disk diffusion methods, antimicrobial gradient diffusion tests, and broth dilution techniques as those recommended by the Clinical and Laboratory Standards Institute or by the European Committee on Antimicrobial Susceptibility Testing .[1,2] However, results obtained by these techniques are time consuming, needing at least 20 h, or up to a month in case of tuberculosis. During the last decades, automated and semiautomated devices, such as: Becton Dickinson Phoenix, Siemens MicroScan Walk Away or Vitek 2, have been extensively used at hospital level.[3–5] Nevertheless, they are currently used for the AST determination of fast-growing organisms, have a high cost and, in addition, are not available for the full spectrum of bacteria.

Although it has been reported that antibiotics exert specific effects on growing microorganisms, few studies have attempted to quantify the dynamic changes induced by antibiotics in microbial growth patterns.[6] We previously reported that atomic force microscope based nanomotion sensors can be used to characterize metabolic activity of living bacteria within minutes, needing only 10^2 bacteria to have a measurable signal (**Figure 1**).[7–9] Furthermore, we have described the use of such a device to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ampicillin, and the effects of kanamycin, ciprofloxacin, and caspofungin, for different kinds of fast-growing organisms, such as *Escherichia coli*, *Staphylococcus aureus,* and *Candida albicans*.[7,8] Nevertheless, it has not yet been determined whether this device is sensitive enough to detect cantilever fluctuations produced by slow-growing bacteria (SGB), when incubated in

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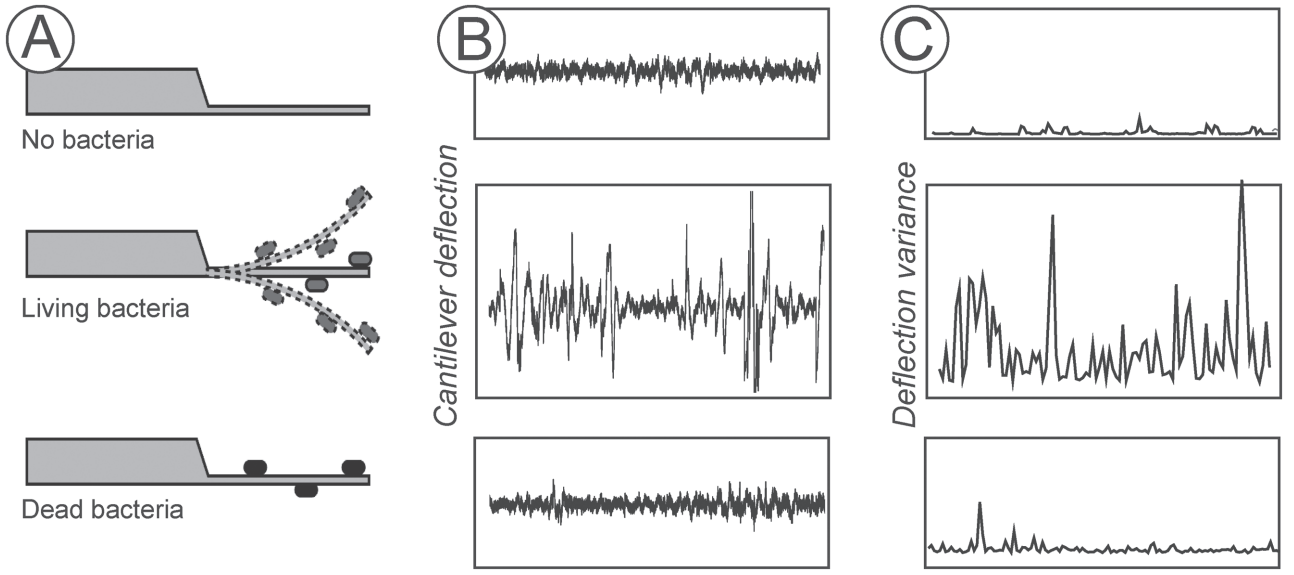

Figure 1. Principles of nanomotion technique. A) To a chemically functionalized cantilever, bacteria are attached. If metabolically active, they will induce oscillations of the cantilever, measured by an optical lever detection method. Once the cells are exposed to antibiotics that inhibit their activity, cantilever oscillations cease. B) Oscillation signal that corresponds to cases of cantilever with no bacteria (top), living bacteria attached (middle), and bacteria exposed to antibiotics (bottom). C) Oscillation signal's variance in time for the same cases.

culture media and in the presence of antibiotics. In this work, we explored the nanomotion sensor's ability to determine the MIC and MBC of macrolides antibiotics in SGB.

We have previously assessed the growth kinetic of *Bordetella pertussis*, the etiological agent of whooping cough or pertussis, in both Stainer–Scholte (SS) liquid medium as planktonic cells, and adhered to surfaces as sessile populations forming biofilm.[10,11] *B. pertussis* can adhere and live attached to different surfaces (polypropylene, glass) where it shows a specific growth rate (μ) of 0.03 h^{-1}.[11] This growth rate is very slow compared to the one of *E. coli* (MG1655), which displayed a μ value of 1.09 h^{-1}, growing on polyethylene terephthalate surfaces.[12] Accordingly, we decided to use *B. pertussis* as a representative organism of SGB in order to assess the impact of different antibiotic concentrations on its metabolic activity and to evaluate the time nanomotion detection requires to sense susceptibility to different antibiotics. Macrolides such as erythromycin or clarithromycin are regarded as antibiotics of choice in the treatment of pertussis. Cotrimoxazole (1:19 trimethoprim/sulfamethoxazole) is often cited as an alternative where macrolides cannot be tolerated.[13] In this work, we have focused on bacteriostatic antibiotics, for which bacteriostatic activity has been defined as an MBC/MIC ratio >4.[14] Finally, the results obtained with the nanomotion sensor for AST analysis were compared with the ones coming from traditional techniques as broth dilution method.[1]

2. Results

The standard method of broth dilution was used to evaluate the MIC and MBC values for *B. pertussis* Tohama I reference strain and the clinical strain Bp2723 grown as planktonic cells (Table S1, Supporting Information). We found that the MIC values of planktonic cells for erythromycin and clarithromycin were between 0.06 and 0.12 μg mL^{-1} and the MBC between 2.5 and 5.0 μg mL^{-1}. The respective MBC/MIC ratios were greater than four for erythromycin, clarithromycin, and cotrimoxazole. Thus, these antimicrobials could be considered as bacteriostatic.[14] We found that the MIC value for each antibiotic tested was the same for both the clinical strain and the reference strain, grown under planktonic condition. Similar results were found for the MBC values of the two strains. Tohama I strain showed MIC and MBC values of 3 and 10 μg mL^{-1} of streptomycin, respectively, while BPSM strain survived the exposure to 200 μg mL^{-1} of the same drug (Figure S1, Supporting Information). In the first set of experiments, nanomotion measurements were done on *B. pertussis* Tohama I reference strain in SS liquid media. The aim of the measurement was to assess the survivability of bacteria on a cantilever. Therefore, the cantilever oscillations were monitored during 13 h of incubation under nonstressing conditions. After 6 h from the start of the experiment, the oscillations continuously increased with time. This phenomenon could be associated with the beginning of replication of the adhered cells, since they are entering the exponential phase of growth, as we have previously reported for *B. pertussis* biofilm growth.[10,11] Bacterial viability after attachment procedure was also assessed using fluorescent dyes (Figure S2, Supporting Information). **Figure 2** shows the variance of the cantilever oscillations during the time course of the experiment. In further experiments, we explored cantilever oscillations after the antibiotic exposure. We chose clarithromycin's and erythromycin's MBC values previously determined by the broth dilution method (Table S1, Supporting Information) to test the outcome on metabolic activity of *B. pertussis* Tohama I strain during 3 h of incubation. Reduction of the sensor's oscillation amplitudes and of the corresponding variance took place after the antibiotic exposure (**Figure 3**). We

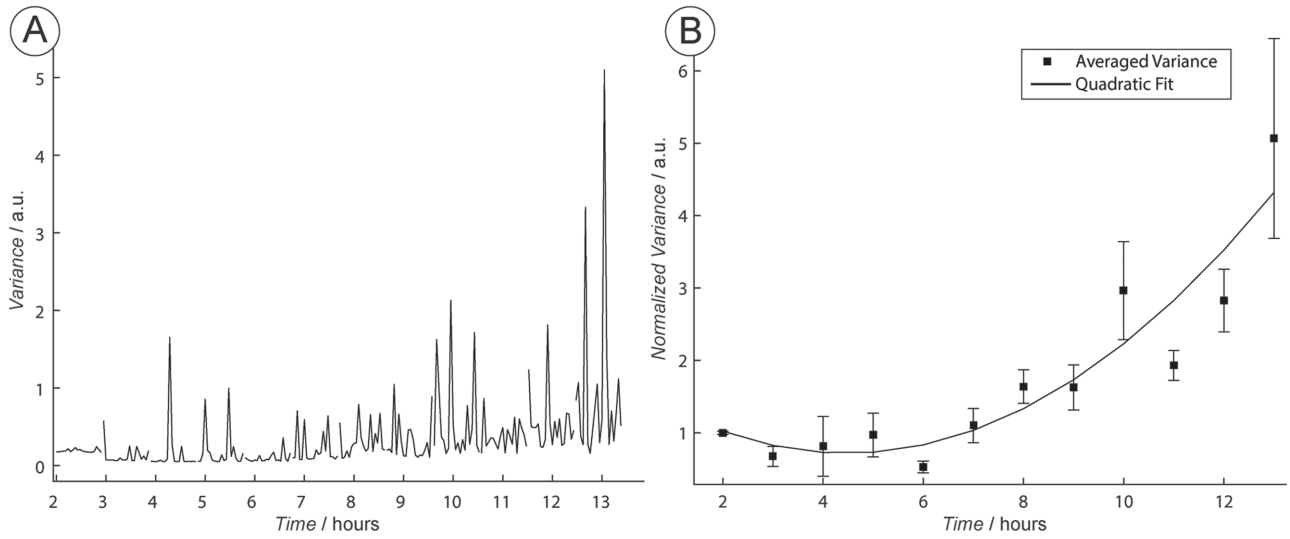

Figure 2. Variance of the cantilever oscillation signal in time. *B. pertussis* cells were attached to the cantilever and tracked during 13 h of incubation in SS media. A) Line plot of the variance against time. B) Variance averages of 1 h window better reflect the increase in oscillations throughout time.

have observed that 40 min incubation time is sufficient to register the effect of the clarithromycin and erythromycin, and less than 20 min for ampicillin (Figure S3, Supporting Information). This result is in agreement with those previously reported with this device but using fast-growing bacteria.[15]

In addition, we monitored the cantilever oscillations when *B. pertussis* BPSM, a streptomycin-resistant (Smr) strain resistant to streptomycin, was incubated in presence and absence of specific antibiotics. The variance was higher for BPSM in SS medium with the addition of streptomycin at 50 μg mL^{-1}, indicating that the metabolic activity was not reduced. The variance of the signal decreased significantly when the bacteria were exposed to SS medium with cotrimoxazole, which suggests a drop in metabolic activity in such environment (**Figure 4**).

To monitor the effect of different antibiotic concentrations on the reference strains and the clinical isolate, we incubated each strain in SS medium alone and in presence of increased antibiotic concentrations from 0.002 to 10.0 μg mL^{-1}. Macrolide clarithromycin and β-lactam ampicillin were employed for this set of experiments. The used concentration range includes MIC and MBC values. Surprisingly, we registered that the larger variance values correspond to the MIC in cases of clarithromycin. The metabolic activity of *B. pertussis* cells adhered to the cantilever increased when it was exposed to antibiotic concentrations lower than MIC in all cases. Culture experiments suggest high viability of the attached cells at MIC values (Figure S4, Supporting Information). After reaching MIC, the variance values drastically decreased as the concentration of antibiotic in the culture medium was increased. This behavior was observed until the antibiotic concentration reached MBC value. Above MBC point, the variance of the oscillation signal (which reflects the cellular metabolic activity) dropped to the lowest values measured in the experiment (**Figure 5**). Finally, we compared the MIC and MBC concentrations obtained with the device with the ones obtained by the conventional method and we found similarities between these two methodologies using the clinical strain and *B. pertussis* reference strain.

3. Discussion

First set of experiments measuring living *B. pertussis* cells on a cantilever during a prolonged period of time confirm the sensitivity of the device to register small signal changes originating from the increase in metabolic activity (from lag to log phase) and multiplication. In further experiments, we showed that a decrease in variance of the signal, compared to the one in growth media, suggests the action of clarithromycin and erythromycin on *B. pertussis* cells on the sensor. In case of resistance to streptomycin, the signal's variance does not decrease with the addition of the drug; however, on incubation with cotrimoxazole, the signal's variance reduction is apparent.

As in case of resistance (BPSM strain's response to streptomycin), the high variance before and close to MIC values is possibly a consequence of the stress condition imposed by the presence of antibiotic in the cellular environment. This observation is consistent with previously reported studies. Compensatory response to antibiotics has been observed in *Pasteurella multocida* under sub-MIC conditions.[16]

4. Conclusion

The above results confirm that the nanomotion sensor not only detects the metabolic activity of an SGB like *B. pertussis*, but also permits to determine the MIC and MBC values in a shorter time than the traditional methods. It constitutes a great advantage for the SGB infectious diseases treatment. Such a rapid antibiotic susceptibility test could reduce treatment costs and more importantly diminish health risks. The new strategy for detecting antimicrobial resistance would be particularly useful

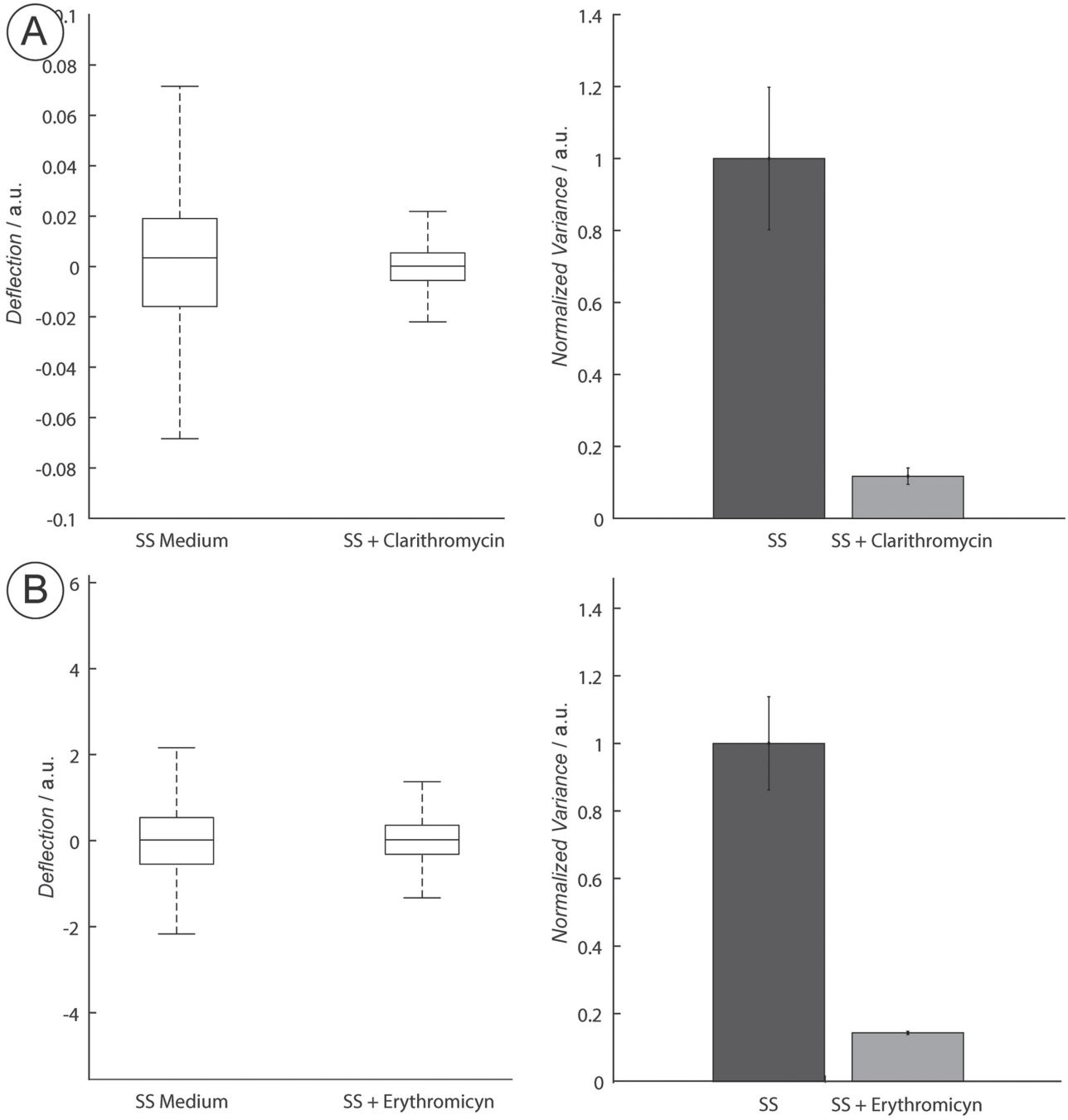

Figure 3. A) Boxplots of the cantilever oscillation movement with *B. pertussis* cells adhered and incubated in SS medium with and without the addition of clarithromycin (5 μg mL^{-1}). B) Normalized variance averages across 15 min of incubation time (15 min averages were taken after 40 min of incubation in the medium or antibiotic).

in cases of infectious disease where the treatment lasts several weeks or months, thus helping to avoid the illness recurrence.

5. Experimental Section

B. Pertussis Tohama I strain (Collection of Institute Pasteur, Paris, France -CIP 8132-): BPSM, an Smr strain derivative from *B. pertussis* Tohama I[17] and *B. pertussis* clinical strain (Bp2723) collected at La Plata Children's Hospital (Hospital Sor Maria Ludovica, La Plata, Argentina) were employed throughout this study. The antibiotics used in the experiments were: erythromycin (Sigma- E6376); clarithromycin (Sigma A3487), trimethoprim-sulfamethoxazole, also known as cotrimoxazole, 1:19 (Sigma T7883 and Sigma S7507, respectively) [TMP-SMX], and ampicillin (Sigma- A0166). In the case of BPSM strain, streptomycin (Sigma S6501) was employed. SS liquid medium was used to culture *B. pertussis* strains.[11,12] Bacteria were deposited onto triangular silicon

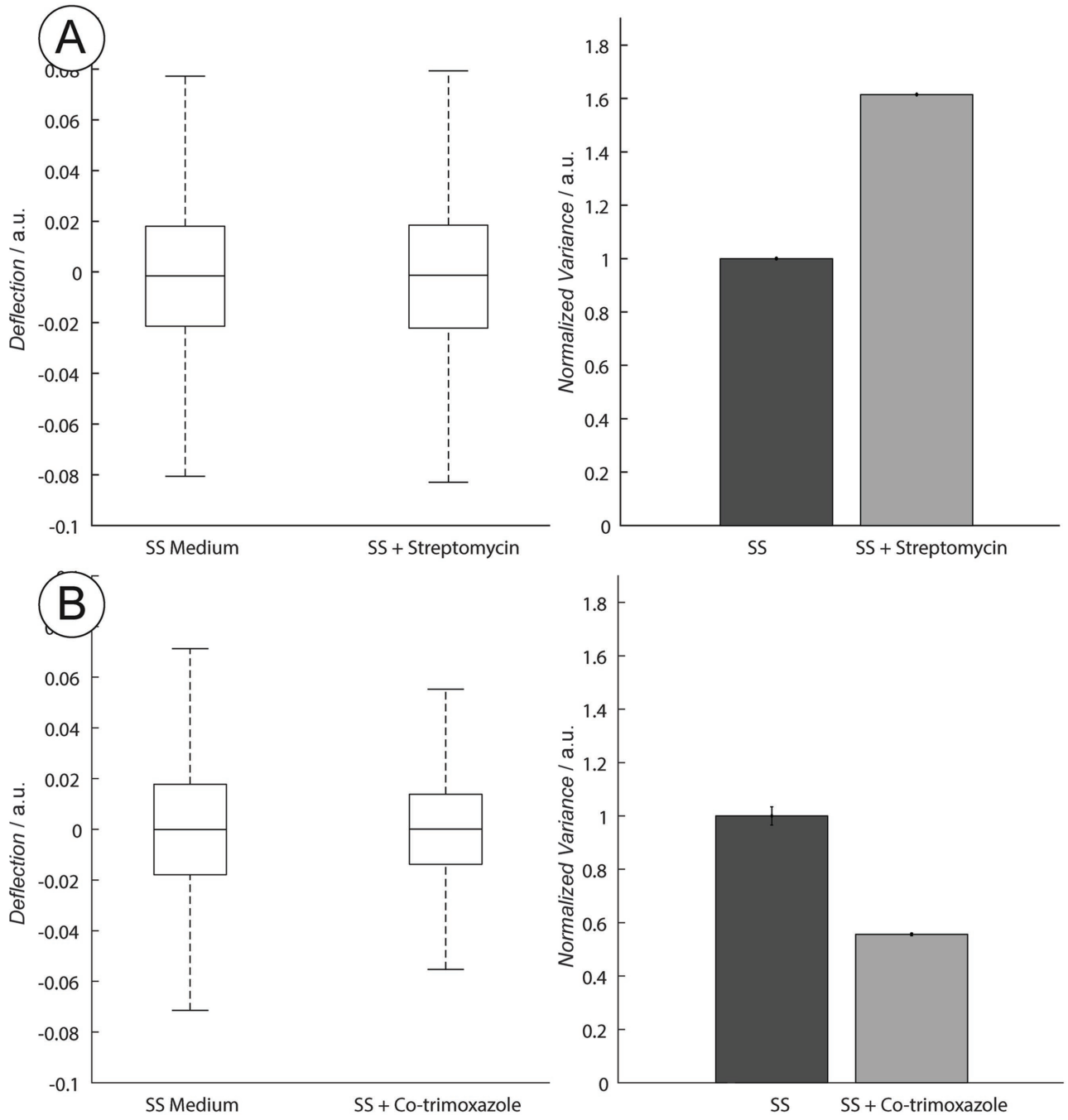

Figure 4. Boxplots of cantilever movement with BPSM (streptomycin resistant) strain adhered and their corresponding variance averages. A) After the introduction of streptomycin, boxplots suggest higher oscillation amplitudes and variance bars confirm an increase in the movement. B) Signal comparison between the exposure to medium and cotrimoxazole. Boxplots show a decrease in oscillation amplitudes, and variance bars support the same conclusion. The responses are connected with A) resistance and B) susceptibility of bacteria to the applied antibiotics. Variance bars are 15 min averages, taken after 40 min of incubation in the medium or the antibiotic.

nitride (Si_3Ni_4) cantilevers with spring constants typically 0.06 or 0.12 N m^{-1}. Before deposition, in order to promote bacterial attachment, the cantilevers were incubated with 10 µL of 0.5% glutaraldehyde for 10 min, rinsed with ultrapure water, dried, and then incubated with 10 µL of a high-density bacterial suspension (OD_{595}: 0.5 with 100 µL of suspension diluted in 1 mL of phosphate buffer saline). The cantilevers with adhered bacteria were inserted into the analysis chamber of a homemade nanomotion device to analyze their vibrational response in growth medium and upon exposure to antibiotics.[15] For each tested condition, 40 min measurements of the cantilever oscillations were done at the room temperature. Variance of the deflection signal was calculated to define the variation in the amplitude of the sensor's movements. The evident outliers were removed in cases of inevitable nonbiological signal. The information was recorded using custom software optimized

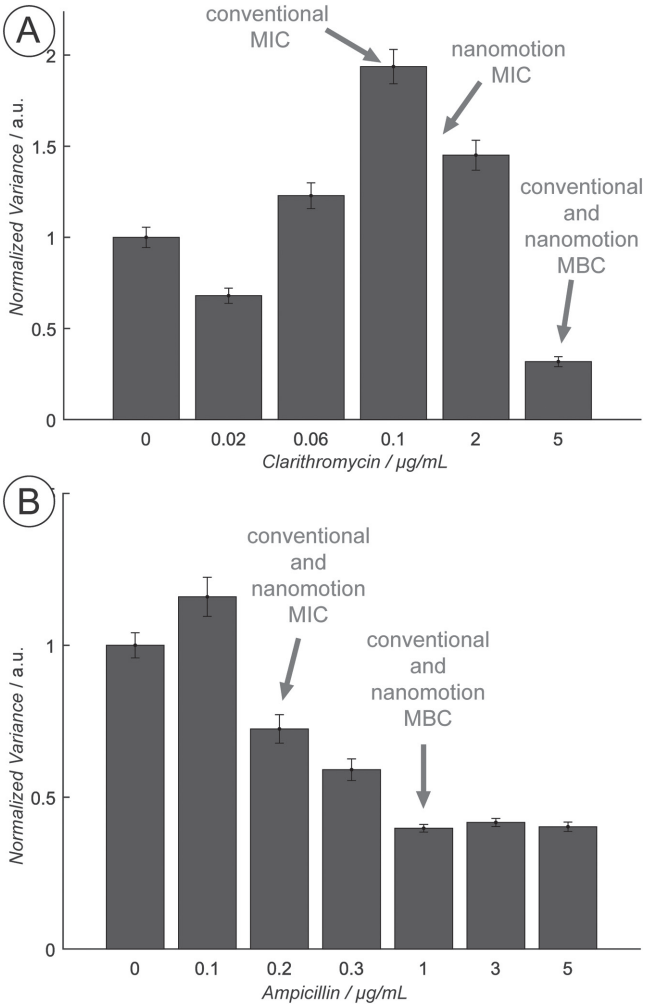

Figure 5. Normalized variance of the cantilever movement obtained when *B. pertussis* was exposed to different concentrations of A) clarithromycin and B) ampicillin. MIC and MBC values obtained by the conventional methods in parallel are marked in red. Results suggest a potential application of such a method for obtaining MIC and MBC values in a much shorter time frame. Each bar corresponds to 30 min average. Considering that clarithromycin and erythromycin need at least 40 min of exposure for an effect to take place, the variance average is shown to be higher, whereas in case of ampicillin, the MIC action takes place within the variance average and the resulting value at MIC is lower that the condition without the antibiotic.

for this application to register the cantilevers movement, and the Matlab R2013b software was employed to analyze the data. For a summary of data presentation approach, please refer to Figure S3 in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

ast, afm, nanomotion, slow-growing bacteria

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