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Proton Nuclear Magnetic Resonance Spectroscopy as a Technique for Gentamicin Drug Susceptibility Studies with *Escherichia coli* ATCC 25922

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Antimicrobial drug susceptibility tests involving multiple time-consuming steps are still used as reference methods. Today, there is a need for the development of new automated instruments that can provide faster results and reduce operating time, reagent costs, and labor requirements. Nuclear magnetic resonance (NMR) spectroscopy meets those requirements. The metabolism and antimicrobial susceptibility of *Escherichia coli* ATCC 25922 in the presence of gentamicin have been analyzed using NMR and compared with a reference method. Direct incubation of the bacteria (with and without gentamicin) into the NMR tube has also been performed, and differences in the NMR spectra were obtained. The MIC, determined by the reference method found in this study, would correspond with the termination of the bacterial metabolism observed with NMR. Experiments carried out directly into the NMR tube enabled the development of antimicrobial drug susceptibility tests to assess the effectiveness of the antibiotic. NMR is an objective and reproducible method for showing the effects of a drug on the subject bacterium and can emerge as an excellent tool for studying bacterial activity in the presence of different antibiotic concentrations.

Infectious diseases remain a challenge in terms of mortality and morbidity despite the great progress achieved in public health in recent decades. For this reason, antimicrobial susceptibility evaluations and new antibiotic screenings are a field of great importance (1). Antimicrobial susceptibility testing (AST) studies are crucial to determine the most effective drug available for the treatment of a patient with an infectious disease process. Currently, automated *in vitro* AST systems are used with standardized methods and conditions to determine the MIC of a drug. These tests usually function by visually judging liquid turbidity, and are one of the reference methods still used today. Unfortunately, these tests involve multiple time-consuming steps, which are often slow, laborious, and susceptible to testing sensitivity problems (2, 3). Moreover, these methods should be useful against pathogens whose susceptibility to drugs is not predictable, thus enabling researchers to determine the antimicrobial susceptibility of fastidious organisms (4) and of those microorganisms that have acquired resistance mechanisms. Measuring the susceptibility of microorganisms to antimicrobials began in the early 1920s (5). Subsequently, there was recognition (as early as the late 1950s) that required the standardization of these techniques. Therefore, numerous AST methods have been described (6). However, there is currently a need for the development of new automated instruments that can provide faster results, reduce reagent cost, and minimize labor requirements (7, 8). In this context, several methods were recently established to minimize the time required to complete the test and the number of operation steps (9, 10).

Nuclear magnetic resonance (NMR) spectroscopy has been used as a method to determine structures of synthesized and natural compounds and to analyze the tridimensional structure of biomolecules. The origin of the NMR spectroscopy technique dates back to 1946; however, it was not until the 1970s when NMR spectroscopy was utilized during metabolic studies (11, 12). Subsequently, in the last few years, NMR spectroscopy has been used for many applications in biology (13) fields, including food science (14–17), pharmacy, and medicine (18). Within these areas, NMR spectroscopy features in the study of metabolome (19). In this sense, metabolites serve as direct signatures of biochemical activity and the behavior of cells. Thus, the terms metabolomics/metabonomics emerged and were defined as the systematic study of the chemical fingerprints of specific cellular processes (19). NMR technology is based on using the magnetic properties of atomic nuclei. Although, a detailed description of the theory and fundamentals of NMR would detract from the objective of this paper, we will describe some aspects of the technique that can help in understanding the equipment and methodologies used in metabolic studies. To obtain an NMR signal, the sample is irradiated with a radiofrequency, which causes it to reach the state of resonance. The resonance of the excited nuclear magnets is detected as an oscillating current in a coil placed around the sample. This signal is the free induction decay, which arrives at the receiver and provides a spectrum formed by lines that define frequencies and widths by a Fourier transformation. A line in the NMR spectrum obtained at a certain frequency (or chemical shift) corresponds to an atomic nucleus with a given chemical environment, which provides structural information about the molecule it belongs to (17).

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Escherichia coli is one of the main causes of nosocomial infections in humans, and the selection of antibiotic resistance mechanisms in its pathogenic and nonpathogenic isolates are associated with the widespread use of antibiotics (20). This work focuses on the use of ^{1}H NMR spectroscopy compared with a broth macrodilution reference method to study the antimicrobial susceptibility of *E. coli* ATCC 25922 (21).

MATERIALS AND METHODS

Organism. The bacterial strain used for the current study was *E. coli* ATCC 25922.

Antimicrobial agent. A stock solution of gentamicin pure powder (Sigma-Aldrich Co., St. Louis, MO) was made up in sterile water. Serial dilutions of the drug (0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) were prepared.

Broth macrodilution MICs. The isolation of bacteria was carried out on brain heart infusion (BHI) agar (Panreac Química S.L.U., Barcelona, Spain) to check bacterial viability at 37°C for 24 h. After that, reisolation was performed to ensure a pure culture in the same conditions. The inoculum of bacteria was prepared to obtain a suspension with a turbidity of a 0.5 McFarland standard, using the direct suspension method. For this, the bacterial suspension was prepared in 5 ml of sterile saline solution (NaCl 0.9%) by matching its turbidity to a 0.5 McFarland standard resulting in a viable bacterial count of ~10^8 CFU/ml. MICs were determined by the reference macrodilution method of the Clinical and Laboratory Standards Institute (22). Samples and control solutions were prepared in a total volume of 2 ml containing bacterial suspension, antimicrobial gentamicin, and Mueller-Hinton medium (Becton Dickinson Microbiology Systems, Sparks, MD). Serial dilutions of gentamicin were tested. A concentration of 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, or 64 μg/ml was added into each tube. Control I was the Mueller-Hinton medium with an inoculum of bacteria prepared under similar conditions as the samples but without an antibiotic, and control II was only Mueller-Hinton medium. All samples and controls were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the drug that completely inhibited visible bacterial growth. For the viable cell count, culture plates were prepared with BHI agar medium. From samples and controls, different dilutions were prepared into sterile water and uniformly spread with sterile spreaders on the plates. All plates were incubated at 37°C for 24 h, and after that time, bacterial colonies were counted manually. The total numbers of bacteria in each sample and control I were determined from the average colony count obtained from different dilutions.

Broth macrodilution samples for NMR spectroscopy. After incubation for 24 h, specimens of 0.540 ml from each sample and control were transferred into 5-mm NMR tubes (5-mm high-precision NMR sample tubes, Norell Inc., Mayslanding, NJ). Furthermore, 60 μl of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) (sodium salt; Sigma-Aldrich Co., St. Louis, MO) diluted in deuterated water (D_2O; deuterium oxide, Euriso-top, Saint-Aubin, France) to a final concentration of 1.125 mg/ml was added as an internal standard.

^{1}H NMR experiments. The equipment used for the acquisition of the spectra was a Bruker Avance 400 spectrometer UltraShield (Avance II 400 MHz, Bruker Corporation, Regensburg, Germany) equipped with a BBI H-BB Z-GRD probe, specifically for proper acquisition of the proton. Spectra acquisition was carried out with the program TOPSPIN version 2.1 (Bruker Corporation, Regensburg, Germany). Processing of spectra was carried out under the program MestReNova version 8.1 (Mestrelab Research, Santiago de Compostela, Spain). Before spectra acquisition, the magnetic field was adjusted for the mix H_2O-D_2O, and all experiments were performed at 25°C. ^{1}H NMR spectra were recorded with a pulse sequence signal presaturation of water located at 1,875 Hz using the pulses zgpr program. For a large and quantitative experiment, we used a relaxation time (d1) of 10 s and a number of scans (ns) of 64 to give a total acquisition time of 20 min. For a short experiment, we used a d1 of 2 s and an ns of 8 to give a total acquisition time of 3 min.

Direct incubation of *E. coli* isolates into an NMR tube as incubation reactor and NMR spectroscopy. The inoculum of bacteria was prepared from a suspension of a 0.5 McFarland standard using the direct suspension method. From a total volume of 2 ml containing the bacterial suspension in Mueller-Hinton medium (with and without gentamicin), 0.540 ml was transferred to 5-mm NMR tubes. Furthermore, 60 μl of TSP diluted in D_2O to a final concentration of 1.125 mg/ml was added as an internal standard. NMR experiments were registered at 37°C every 20 min.

All the experiments were carried out in triplicate.

RESULTS

Stack plots of one-dimensional spectra of incubated bacterial media of *E. coli* ATCC 25922 with gentamicin and spectra of control media (control II) and control of the incubated media without an antibiotic in the presence of bacteria (control I) are shown in Fig. 1.

The comparison of the two control ^{1}H NMR spectra showed different signals in the range of 1.0 to 2.5 ppm. Succinic acid, acetic acid, and ethanol were only detected in the control I spectra and threonine was only detected in the control II medium. These signals were assigned through comparison of their chemical shifts with those reported in the literature (16) and by comprising the spiking with the corresponding compounds in the control samples.

When we registered antibiotic spectra at different concentrations, we detected the presence of succinic acid, acetic acid, and ethanol only in samples with concentrations of gentamicin lower than 0.5 μg/ml. Moreover, when the concentration of gentamicin was greater than 0.5 μg/ml, we detected the presence of threonine (Fig. 1).

According to the results obtained by visual turbidity, the lowest concentration of drug that completely inhibited visible growth (MIC) was 0.5 μg/ml (MIC, 0.25 to 1 μg/ml) (22). These data suggested that the results obtained by ^{1}H NMR spectroscopy were in agreement with those obtained by visual turbidity. A viable count of culture plates showed no bacterial growth on plates with gentamicin concentrations from 64 to 1 μg/ml. However, the plate containing 0.5 μg/ml of gentamicin showed a viable bacterial number in the range of 10^6 CFU/ml. The remaining samples (0.25 to 0.06 μg/ml, including control I) demonstrated a viable bacterial count in the range of 10^{10} to 10^{11} CFU/ml. Data extracted from the counts of viable bacterial growth on the agar plates allowed us to obtain the minimal bactericidal concentration (MBC), that is, the lowest concentration of gentamicin that kills the bacteria. In this case, MBC was observed at 1 μg/ml.

Figure 2 shows the time course evolution of peak areas corresponding to metabolites (succinic acid, acetic acid, and ethanol) or nutrient (threonine) as a function of antimicrobial gentamicin concentration for the *E. coli* strain used. The results obtained from spectra showed that *E. coli* is able to metabolize components of the medium to produce succinic acid, acetic acid, and ethanol. The intensity of these signals varied according to the viable bacteria (CFU/ml) present in the sample, indicating that the intensity was higher in the samples with concentrations of gentamicin lower than the MIC (0.5 μg/ml) in the rest of the samples. Furthermore, threonine only appeared in the spectra of those samples with gentamicin concentrations of ≥0.5 μg/ml.

We were also able to optimize the acquisition time of the NMR experiments. We spent only 3 min on each experiment, conse-

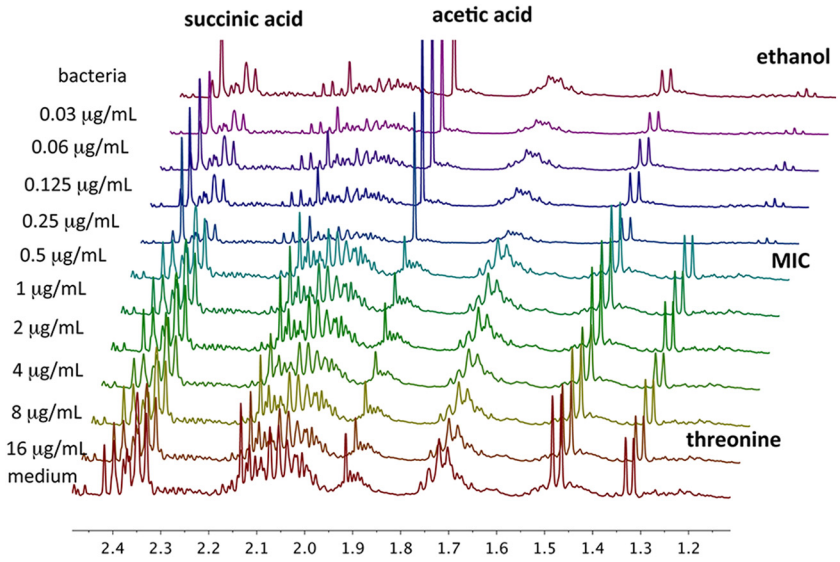

FIG 1 [1]H NMR spectra of samples of *E. coli* ATCC 25922 incubated with gentamicin (concentrations are shown on the left). Control I (growth media with bacterial inoculum) appears at the top of the spectra. Control II (only growth media) appears at the bottom of the spectra. Changes in concentration of metabolites are well shown in spectra (threonine of medium was consumed when bacteria were active; succinic acid, acetic acid, and ethanol were the result of metabolic production of bacteria).

quently decreasing the analysis time considerably without information loss.

To evaluate the potential of this tool, we carried out the same biological experiments but, in this case, using an NMR tube as the incubation reactor. We introduced into the NMR tube the infected culture medium, previously prepared, and registered NMR experiments at 37°C every 20 min. We observed that the ethanol signal appeared at 3 h 40 min while the disappearance of the threonine signal occurred at 5 h 20 min (Fig. 3A). From this data, we can confidently establish that bacterial activity occurred effectively within the NMR tube, and the metabolic process started around 3 h 20 min and ended at 6 h.

Thus, we introduced the infected culture media supplemented by several gentamicin concentrations, previously prepared, into the NMR tubes. Stacked plots of the one-dimensional spectra of incubated bacteria in the NMR tube corresponding to concentrations of 0.06 and 0.125 μg/ml are shown in Fig. 3B and C, respectively. The ethanol signal appeared later using 0.06 μg/ml of gentamicin (4 h 40 min) compared with the experiments performed in the absence of the antibiotic (3 h 20 min) and much later (8 h 40 min) when the gentamicin concentration used was close to MIC (0.125 μg/ml). Similarly, threonine consumption by bacteria was delayed when the concentration of antibiotic in the medium was higher.

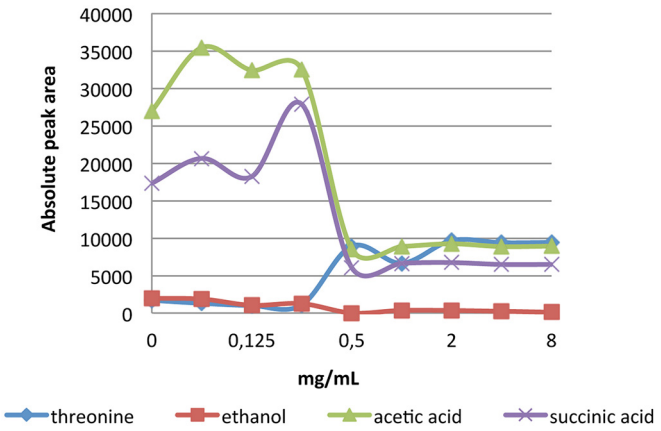

FIG 2 Plots of peak areas corresponding to metabolites or nutrients as a function of antimicrobial gentamicin concentration used for the *E. coli* strain. Acetic acid, succinic acid, and ethanol were produced by bacteria. Threonine is consumed by *E. coli*. When the concentration of gentamicin was higher than 0.5 μg/ml, neither consumption nor production of any metabolites were detected, suggesting that bacteria were not viable.

DISCUSSION

E. coli is a facultative aerobic bacterium able to ferment sugars into a mixture of different compounds, such as acetic and succinic acids, among others, and ethanol by a mixed-acid type fermentation (Fig. 1) (23, 24). Differences in peak intensities for these metabolites observed in spectra allowed us to determine the MIC of gentamicin using NMR spectroscopy. Consumption of the amino acid threonine, present in the culture medium, was interrupted when MIC was achieved. It is well-known that threonine is involved not only in the metabolism of *E. coli* but also in its regulation, and it is one of the amino acids that bacteria quickly catabolize (25, 26). Therefore, we assume that succinic acid, acetic acid, and ethanol are metabolites produced by bacteria and threonine is an amino acid consumed by *E. coli*. These data indicate that microbial activity ceases when the concentration of antibiotic is 0.5 μg/ml.

Although the time taken to perform each experiment (NMR spectroscopy and reference method) was the same, the use of NMR allowed us to observe the bacterial metabolism, ensuring the inviability of the bacteria when MIC was reached. Furthermore, the use of NMR offered us the possibility to determine the viable bacterial count faster than using the reference method. With our results, we can firmly conclude that NMR is able to detect MIC

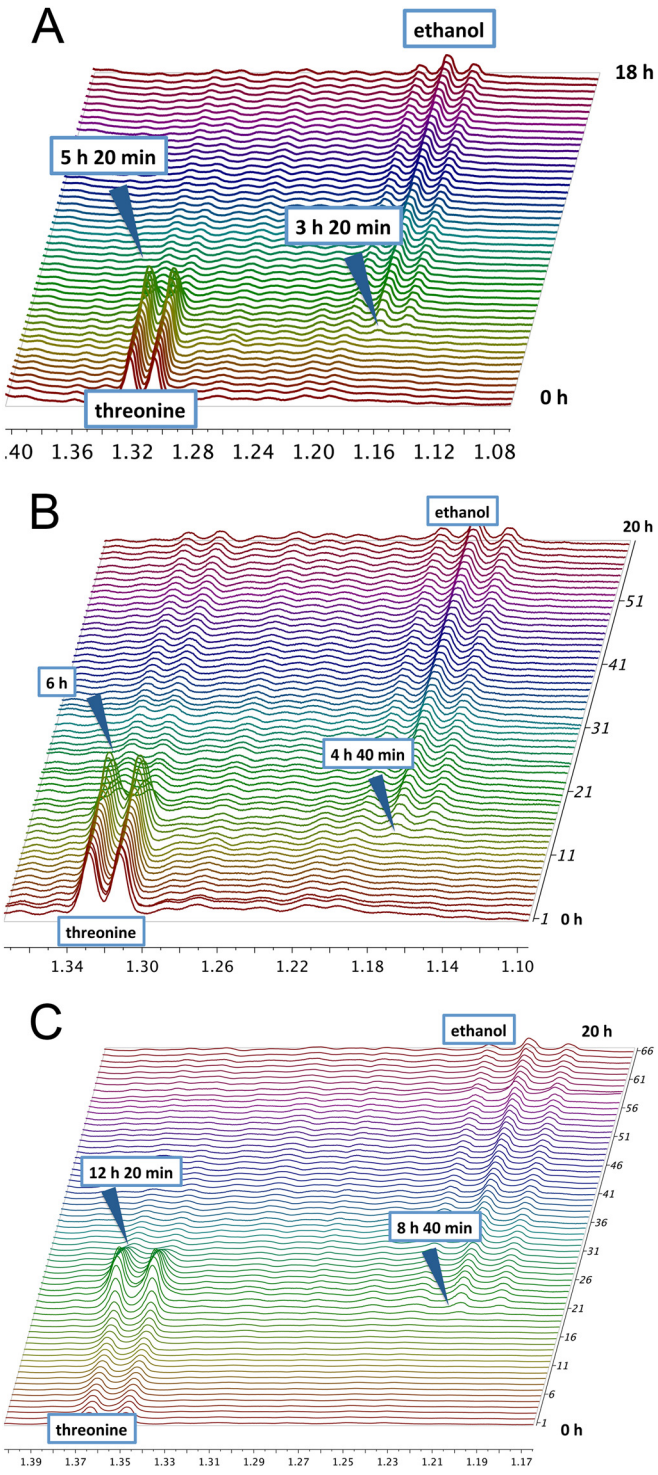

FIG 3 (A) ^{1}H NMR spectra stacked plot of incubated *E. coli* ATCC 25922. Changes in concentration of metabolites are well shown in spectra. Threonine was consumed when bacteria were active, and ethanol is a metabolite resulting from metabolic production of bacteria. (B) ^{1}H NMR spectra stacked plot of incubated *E. coli* ATCC 25922 for 20 h at a concentration of 0.06 μg/ml of gentamicin. (C) ^{1}H NMR spectra stacked plot of incubated *E. coli* ATCC 25922 for 20 h at a concentration of 0.125 μg/ml of gentamicin.

correctly. Although the viable count of the plates showed bacteria in the 0.5 μg/ml agar plate (range of 10^6 CFU/ml), ^{1}H NMR did not detect bacterial metabolism at this range or at MBC. This result suggests that the range of 10^6 CFU/ml may be the limit of detection for the NMR spectrometer used or the limit in which the population of bacteria activates the metabolism.

The experiments carried out using the NMR tube as an incubation reactor enabled the development of the AST experiments directly in the NMR tube to assess the effectiveness of the antibiotic. This NMR technique can become a useful tool for studying bacteria that do not grow on habitual media, whose metabolism is not well known, and for those bacteria that standardized antimicrobial susceptibility tests are not available.

There is a complex relationship between concentrations of antibiotic and growth and death rates of bacteria. This functional relationship, which is called pharmacodynamics, is an important parameter in the rational design of effective antibiotic treatment protocols (27). The bacterial behavior throughout several concentrations of antibiotic can usually be examined by killing or growth curves, which enables the obtainment of additional information regarding antimicrobial properties of antibiotics *in vitro*, particularly with regard to their initial bactericidal activity against different pathogens (28). Killing or growth curves can be used to study anti-infective effects in *in vitro* models, with the advantage of providing more detailed information about the time course of antibacterial effect (29). As we have shown in the previous experiment, NMR is an outstanding technique to study the time course evolution of a biological process.

Additionally, the introduction of the infected culture media in the presence of several concentrations of the antibiotic in NMR tubes allowed us to evaluate the kinetic behavior of bacteria in the presence of gentamicin. As we can see in Fig. 3, it is very easy to follow the bacterial growth using NMR spectroscopy. Taking into account that studies using kill or growth kinetics are expensive in terms of time and cost and that they are not likely to be performed in a routine laboratory, this novel approach using the NMR technique might become a powerful tool for studying antimicrobial drugs.

NMR spectroscopy is a suitable tool to be considered an alternative to those methods that show difficulties in accuracy and reproducibility (30). Moreover, recently Gupta et al., using the ^{1}H NMR metabolic approach, identified and quantified *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* isolates in urine samples from patients with urinary tract infections (UTIs) faster than using culture methods. This NMR method of metabolic profile exploration in urine provided an alternative approach for screening and identification of UTI (31–34). These perspectives, along with the study reported in this paper, suggest the great potential of ^{1}H NMR for antimicrobial analysis. In the field of fungi infections, drug-induced inhibition of fungal growth is used in the diagnostic laboratory to predict the therapeutic efficacies of antifungal agents. NMR spectroscopy has emerged as a modern and powerful tool for detecting subtle effects on fungal metabolism. Specifically, in the NMR spectra, inhibition of fungal growth through metabolite changes, such as ethanol, glucose, succinate, or acetate, may be detected (35–37). Our team has initiated more complex studies using NMR with fastidious microorganisms such as *Rickettsia* spp. with promising results (38).

Future application of this NMR tool in microbiology requires

large studies involving metabolite identification, obtaining metabolic profile information, and correlating the data to find predictive models for validation and standardization. Costs of this technique (hardware and software installation) are relatively high, but in the long term they are diminished due to the reduced operating time, cost of reagents, low-maintenance, and the small quantities necessary per sample compared with conventional methods or with methods based on molecular biology. The use of few and inexpensive reagents allow for this technique to be used in clinical laboratories, in a similar way to how it is used for the analysis of food in reference centers. Moreover, the new technology of low-field magnets that can be in the benches of laboratories can provide a great opportunity to introduce the methodology in the daily routine. Further antimicrobial susceptibility studies directly applied to clinical samples can show differences in the results compared with *in vitro* studies where the stability and the composition of the culture medium is usually well known and differs from the organism composition.

In conclusion, the results obtained from this study on the use of ^{1}H NMR in antimicrobial susceptibility analyses indicate that NMR is an objective and reproducible method to show the drug effect on the bacteria studied. MIC determined by the macrodilution method (CLSI) would correspond with the end of the bacterial metabolism observed by NMR, which also predicts the MIC. In addition, we demonstrated that the NMR technique can emerge as an excellent tool for studying the bacterial behavior in the presence of different concentrations of antibiotic. As we have demonstrated, further studies and the automatization of the technique can provide a rapid and reproducible method for studying antimicrobial drug susceptibility and bacterial identification.

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We declare no conflicts of interest.

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