Clinical Microbiology and Infection 23 (2017) 400–405

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Nanomechanical sensor applied to blood culture pellets: a fast approach to determine the antibiotic susceptibility against agents of bloodstream infections

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ARTICLE INFO

Article history:
Received 21 September 2016
Received in revised form
23 December 2016
Accepted 24 December 2016
Available online 3 January 2017

Editor: F. Allerberger

Keywords:
Atomic force microscopy
Bacteraemia
Bacterial pellet
Bacteriology
Cantilever
Diagnostic microbiology
Nanomechanical sensor
Nanomotion susceptibility test
Rapid analysis

ABSTRACT

Objectives: The management of bloodstream infection, a life-threatening disease, largely relies on early detection of infecting microorganisms and accurate determination of their antibiotic susceptibility to reduce both mortality and morbidity. Recently we developed a new technique based on atomic force microscopy capable of detecting movements of biologic samples at the nanoscale. Such sensor is able to monitor the response of bacteria to antibiotic's pressure, allowing a fast and versatile susceptibility test. Furthermore, rapid preparation of a bacterial pellet from a positive blood culture can improve downstream characterization of the recovered pathogen as a result of the increased bacterial concentration obtained.

Methods: Using artificially inoculated blood cultures, we combined these two innovative procedures and validated them in double-blind experiments to determine the susceptibility and resistance of *Escherichia coli* strains (ATCC 25933 as susceptible and a characterized clinical isolate as resistant strain) towards a selection of antibiotics commonly used in clinical settings.

Results: On the basis of the variance of the sensor movements, we were able to positively discriminate the resistant from the susceptible *E. coli* strains in 16 of 17 blindly investigated cases. Furthermore, we defined a variance change threshold of 60% that discriminates susceptible from resistant strains.

Conclusions: By combining the nanomotion sensor with the rapid preparation method of blood culture pellets, we obtained an innovative, rapid and relatively accurate method for antibiotic susceptibility test directly from positive blood culture bottles, without the need for bacterial subculture. **P. Stupar, Clin Microbiol Infect 2017;23:400**

Introduction

Rapid acquisition of a blood culture pellet, coupled with a subculture-independent technique allowing phenotypic determination of the antibiotic susceptibility, could significantly reduce the time to results, greatly benefitting patient care and reducing cost of treatment, especially empiric treatments with broad-range antibiotics. A reduction of this timeline to less than one working day would be very useful, reducing morbidity and mortality as well as the overall costs for healthcare systems [1]. Furthermore, it would allow achieving faster treatments tailored to the patient, which would be more effective and better tolerated, with a reduced negative impact on beneficial bacteria.

To achieve this, we have proposed a new technique to rapidly characterize bacterial resistance to antibiotics [2–4]. This technique

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http://dx.doi.org/10.1016/j.cmi.2016.12.028

is based on the use of nanomechanical sensors [5–7] that oscillate if the attached bacteria are metabolically active. Oscillation detection is obtained using an optical lever method, where a laser beam is reflected off the cantilever and directed towards a detector (Fig. 1). Even among other nanomechanical sensor systems, this technique stands out as one of the most sensitive and fast in the study of bacterial growth and antibiotic susceptibility [8–13]. Nanomotion–antimicrobial susceptibility testing (AST) has several advantages compared to conventional culture-based systems commonly used in microbiology. For instance, it does not rely on bacterial growth; hence it is not negatively impacted by the replication time of the bacteria, and this reduces the characterization of the microorganism's resistance to minutes, compared to hours or days needed by conventional AST systems [14,15]. Furthermore, nanomotion-AST requires a low number of bacteria (approximately 10^2 bacteria) to obtain a measurable signal [3]. Overall, the speed and sensitivity of this technique allows an unprecedented insight on the bacterial response to environmental, chemical or physical stimuli.

Recently we developed an ammonium chloride–based blood culture pellet preparation test, which allows obtaining a high bacterial amount of an isolated strain for rapid downstream characterization [16]. This work demonstrates the feasibility of the previously described combination of bacterial pellets prepared from positive blood cultures [17] and nanomotion for rapid AST on *E. coli,* the most frequently recovered bacterial pathogen [18]. The findings of this study suggest that these two innovative techniques might serve as a versatile system, greatly affecting the field of microbiology. Their combination would ensure a significant reduction of the time needed to characterize bacteria from positive blood cultures to complete analysis in less than 3 hours.

Materials and methods

Reagents and antibiotics

All chemicals, phosphate-buffered saline (pH 7.4), Luria broth, glutaraldehyde and paraformaldehyde and the antibiotics ampicillin, ciprofloxacin, ceftriaxone and cefoxitin, all of analytical grade, were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Bacterial preparation and isolation from blood cultures

Bacterial strains, and resistant and susceptible strains of *E. coli* were either American Type Culture Collection (ATCC) (Manassas, VA, USA) strains or clinical isolates of the bacterial repository of the Institute of Microbiology of the Lausanne University Hospital (Lausanne, Switzerland). The susceptible *E. coli* strain used for this study was ATCC 25922 (minimum inhibitory concentration (MIC) values for ceftriaxone, ciprofloxacin and ampicillin are 0.06, 0.008 and 8 μg/mL respectively). As resistant *E. coli* strain, we used a clinical isolate characterized using the VITEK device and Etest (bioMérieux, Marcy l'Etoile, France) methods, showing MIC values for ceftriaxone, ciprofloxacin and ampicillin as ≥64, ≥4 and ≥256 μg/mL respectively. Working concentrations of the antibiotics were chosen according to the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org) interpretation guidelines to allow discrimination between resistant and susceptible strains.

Bacteria were inoculated together with human blood in anaerobic culture bottles (BACTEC Lytic/10 Anaerobic/F Medium; Becton Dickinson (BD), San Diego, CA, USA), recovered from the transfusion centre of our hospital, in BD blood vials and processed by a BACTEC FX automated blood culture system (BD). This system automatically detects the growth of microorganisms by detecting pH change due to CO_2 production. When detected positive, blood vials were processed in order to rapidly obtain a bacterial pellet; the method is described in detail elsewhere [17]. Briefly, 5 mL of the medium from a positive blood culture was mixed with 40 mL sterile water; after centrifugation (1000 × *g* for 10 minutes), the supernatant was removed, and the remaining blood cells were lysed by adding 1 mL ammonium chloride (0.15 M NH_4Cl, 1 mM $KHCO_3$, pH 7.31) to the bacterial pellet, followed by a second centrifugation step (140 × *g* for 10 minutes) to discard the lysed red blood cells.

Overall, this preparation protocol lasts less than 1 hour and ensures an enriched bacterial pellet that may be characterized. This bacterial pellet was finally resuspended in buffer and tagged with a code in order to ensure a blind susceptibility test with the nanomotion sensor.

Nanomotion-AST

To perform the susceptibility tests, we used both a homemade small, portable prototype device and a commercial JPK Nanowizard III microscope (JPK Instruments, Berlin, Germany) equipped with an Axiovert inverted optical microscope (Carl Zeiss GmbH, Jena, Germany) (Supplementary Fig. S1). The sensors we used in these experiments were commercial atomic force microscope cantilevers (NP-O10; Bruker Daltonics, Billerica, MA, USA). Sensor deflections were measured using the conventional laser-based system

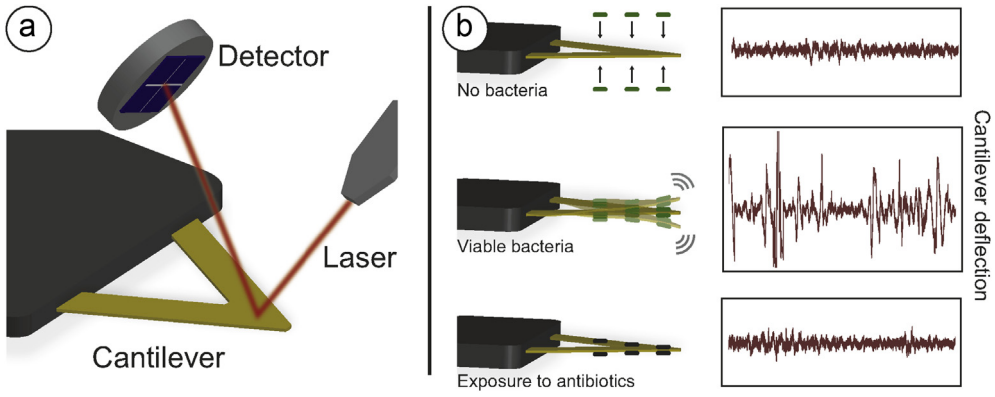

Fig. 1. Outline of experimental setup and description of experiments. (a) Schematics of nanomotion detector setup with cantilever sensor; laser beam is focused on surface of sensor, and reflection is used to monitor movements of cantilever. (b) Representation of typical nanomotion susceptibility test. When bacteria are not attached to sensor, fluctuations are driven only by thermal motion and are low. After attachment of live bacteria, fluctuations are linked to their metabolic activity and are high. Finally, after exposure to bactericidal drug, bacteria are nonviable and fluctuations return to low levels.

common to most atomic force microscopes, which ensures a reliable determination of the movements of the sensor with a sensitivity of less than 0.1 nm. We equipped both instruments with a custom analysis chamber, which was made of polydimethylsiloxane and which allows performing experiments in liquid while changing the medium with very little disturbance [19,20].

The sensors were preliminarily functionalized with 0.5% glutaraldehyde for 7 minutes in order to ensure a firm attachment of bacteria for the time frame of the characterization experiments and were subsequently washed and dried, as previously reported [3]. Once prepared, the treated sensors were readily used to attach the bacteria. We incubated them with 10 μL of the concentrated bacterial suspension for 30 minutes. As a result of high turbidity, the working concentration for optimal attachment was determined by dilution test; namely, resuspending 10 μL of the suspension into 1 mL of phosphate-buffered saline gave an optical density at 600 nm of 0.4 to 0.6, which indicated that the suspension was concentrated enough. Next we gently washed the sensors in growth medium and introduced them in the analysis chamber for characterization. This procedure ensured that the sensor was covered with hundreds of viable and firmly attached bacteria. The chamber was then filled with Luria broth to ensure the viability of the bacteria and to enhance the effectiveness of some antibiotics [21,22]. After 5 minutes of stabilization of the entire system, we started the nanomotion-AST measurements.

We recorded the dynamic fluctuations of the sensor in different conditions and we measured the fluctuations for at least 20 minutes, choosing an acquisition rate of 20 kHz (Supplementary Fig. S2). The first step was to obtain the signal while the attached bacteria were in Luria broth medium. Then we substituted the medium in the analysis chamber with the same one but containing the chosen antibiotic, and continued the recording of the sensor's fluctuations. In this case, we measured for longer time (approximately 60 minutes) to ensure that the antibiotic effect was stable and that the bacteria had completed their response to the exposure of the drugs. In all cases, we collected time-dependent fluctuations of the sensor. The signal was flattened and then filtered using a moving average filter. To evaluate the effective variation in the amplitude of the sensor's movements, the deflection curves were statistically analysed by calculating their variance. If applied in a windowed manner (of 10 seconds), this parameter allows following the evolution of the bacterial dynamics throughout each experiment. At this stage, obvious outliers are removed as few occurrences of nonbiologic signal are inevitable (accidental contact with the device). 'Change' numbers were calculated by averaging variance values. For signal acquisition, we developed a software based on LabVIEW (version 2013; National Instruments, Austin, TX, USA), and for all signal processing purposes, the software was developed in a commercial MATLAB 8.6 package (MathWorks, Natick, MA, USA).

We previously demonstrated that the reduction of the signal is synonymous with susceptibility to the antibiotic tested [2]. We used this readout to categorize susceptible and resistant strains. In order to test the combination of our two methods—the rapid concentration and purification of bacteria from artificially inoculated positive blood cultures and the nanomotion-AST—we performed analyses on *E. coli* as the main blood infection pathogen, including both susceptible and resistant strains for the selected antibiotics. Susceptibility of *E. coli* was tested with ciprofloxacin, ampicillin and ceftriaxone.

Results

The results presented in Fig. 2 correspond to different blind experiments performed on *E. coli* exposed to ampicillin (16 μg/mL), ceftriaxone (2 μg/mL) and ciprofloxacin (2 μg/mL) respectively. Fig. 2 shows typical results from these experiments, presenting cantilever oscillations as the evolution of variance. In all cases, the attached bacteria induced measurable fluctuations of the sensor, that remained stable and indicated the sustained viability of the microbes throughout the experiments. Upon injection of the antibiotic-enriched medium, the fluctuations changed quite rapidly. Different drug–bacteria combinations yielded a change of the signal at different time points. Exposure of susceptible strains to ampicillin produced sharp reduction in the fluctuations 20 minutes after the drug injection, while ceftriaxone and ciprofloxacin required 50 and 40 minutes to produce a stable response.

These experiments were all performed using a double-blind protocol in order to ensure that results would not be influenced by the experimenter's knowledge of the bacterial characterization. The criteria used for classification were based on variance of the sensor's movement (Fig. 2A1, 2B1, 2C1), in order to see whether a reduction occurred. When the blind labels were revealed, the positive identification rate was higher than 94% (16 of 17 correct assessments). Results are provided in Table 1. Once the labels were known, we quantified the results by calculating the change value (%). The value represents the change of variance of the signal occurring when the nourishing medium is replaced by the one containing the antibiotic. Calculation points were taken 40, 50 and 20 minutes after the exposure to ciprofloxacin, ceftriaxone and ampicillin respectively.

Overall, when testing resistant strains, we observed that the variance can surge to very high values (i.e. 190% of the initial signal before the antibiotic) or can drop subtly (i.e. 80%), indicating the variability of bacterial response within the same bacterium–antibiotic conditions. In cases of susceptible strains, the reduction of the fluctuations after the antibiotic exposure was larger (to 10, 24 or 27%).

In Fig. 3 we plotted the overall variance change values presented in Table 1 for each tested sample. The upper limit in variance changes considering susceptible cells is in the sample labelled 102, at 49%. Within the resistant bacteria data set, the lower limit shows up in sample 112, at 78%. On the basis of these experiments, we defined a threshold in change of variance, allowing the classification between susceptible and resistant strains (Fig. 3). A value of 60%, which is the upper boundary determined by the standard error of the uppermost value of susceptible samples, would correctly discriminate the susceptible and resistant strains, including the resistant sample 207. The latter was initially misclassified as susceptible on the basis of the qualitative analysis of the variance graph (Supplementary Fig. S3).

Discussion

Here we have shown an innovative protocol as a combination of the ammonium chloride centrifugation technique to concentrate bacteria from positive blood cultures rapidly, and fast and sensitive nanomotion-AST. To determine the overall time needed to perform a complete characterization, we need to consider the purification of bacteria from the positive blood culture (time to obtain a bacterial pellet) and the time needed to perform the nanomotion-AST. In all our experiments, the elapsed time from determination of the positive blood culture to the end of the susceptibility test was less than 3 hours. These results highlight the importance of combining the two innovative and rapid techniques, as well as the potential of their application to the clinical setting.

Nanomotion-AST is one of the many important applications of the nanomechanical sensor. Such a sensitive system can be important in microbiology in a variety of additional applications. For instance, for slow-growing bacteria such as mycobacteria, this technology may prove transformative. Furthermore, the

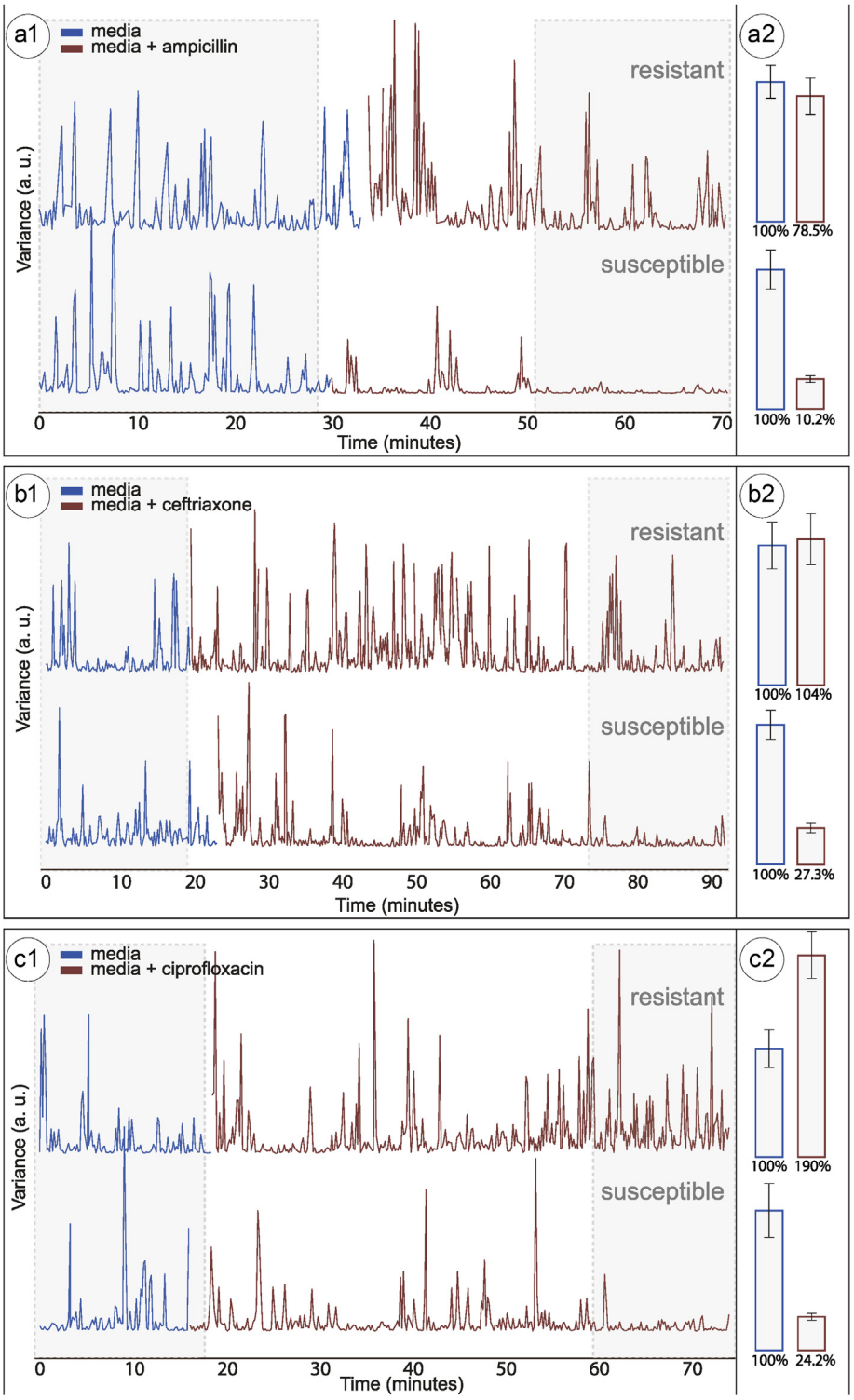

Fig. 2. Variance evolution throughout experiments: effect of ampicillin (a), ceftriaxone (b) and ciprofloxacin (c) on *Escherichia coli*. Typical examples of resistant *E. coli* strain (top) and susceptible strain (bottom) are presented. Blue colour shows variance of sensor's movement while only nourishing media is present in system. Presence of antibiotic and corresponding variance trend is shown in dark red. Area shaded in grey is used for averaging and presenting as change (%) on second panels.

nanomotion-AST analysis can characterize bacterial species as well as many other biologic systems with a versatility and speed unachievable using currently available methods [23,24]. This might allow more information to be extracted on the interaction between bacteria and antibiotics—to assess not only whether an antibiotic has had an effect but also what kind of effect and the dynamics of the bacterial response.

Our work stands as a proof of principle. It needs to be extended with (a) a higher biodiversity of bacterial species, including all 20 species most commonly recovered from blood, (b) a larger range of antibiotics and (c) a variety of strains directly recovered from patient blood, including strains with intermediate antibiotic resistance. Specifically, our data suggest that susceptible and resistant strains could be correctly categorized using a specific threshold for

Table 1
Summary of nanomotion-AST measurements of *Escherichia coli* and their evaluation against conventional technique

Sample ID	Antibiotic	Concentration (μg/mL)	Nanomotion-AST result (before defining threshold)	Correct[a]	% Change[b]
101	Ciprofloxacin	2	Resistant	Yes	184
102	Ciprofloxacin	2	Susceptible	Yes	49
103	Ciprofloxacin	2	Resistant	Yes	190
104	Ciprofloxacin	2	Susceptible	Yes	28
105	Ciprofloxacin	2	Resistant	Yes	88
106	Ciprofloxacin	2	Susceptible	Yes	24
100	Ceftriaxone	2	Susceptible	Yes	27
200	Ceftriaxone	2	Susceptible	Yes	32
107	Ceftriaxone	2	Resistant	Yes	104
207	Ceftriaxone	2	Susceptible	No	80
108	Ceftriaxone	2	Resistant	Yes	86
208	Ceftriaxone	2	Resistant	Yes	97
110	Ampicillin	16	Susceptible	Yes	18
210	Ampicillin	16	Resistant	Yes	99
111	Ampicillin	16	NA	NA	NA
211	Ampicillin	16	Susceptible	Yes	22
112	Ampicillin	16	Resistant	Yes	78
212	Ampicillin	16	Susceptible	Yes	10

AST, antimicrobial susceptibility testing; NA, not applicable (measurement failed due to a technical issue).
[a] Sixteen tests matched correct response.
[b] Change in percentage represents how much signal is present after drug injection compared to measurement in Luria broth. Calculations were done after 40, 50 and 20 minutes of exposure to ciprofloxacin, ceftriaxone and ampicillin respectively.

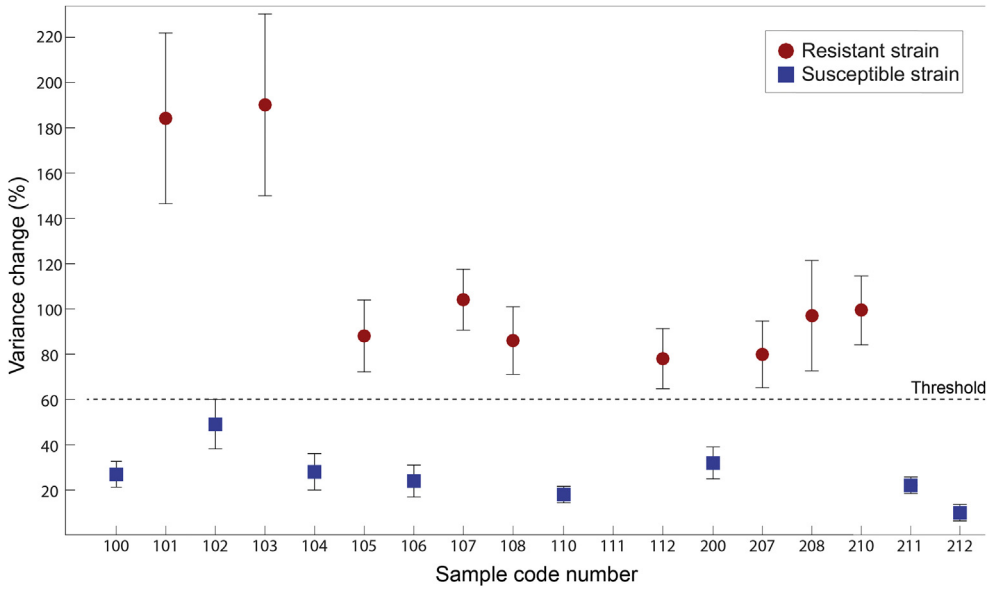

Fig. 3. Plot of change values from Table 1, representing percentage change of oscillatory movements before and after addition of drugs. Susceptible strains fall below 60%, meaning that addition of drug is reducing oscillations for more than 40%. However, resistant strains produced either unchanged movement (100%), increased movement or movement with subtle change (lower limit around 80%). Therefore, threshold value of 60% could be defined as marker for distinguishing susceptible from resistant strains.

change in variance before and after the exposure to a defined antibiotic. In the future, developing a highly parallelized and automated nanomechanical detection system could allow simultaneous rapid AST for various antibiotics generally used to treat a given isolated species. By multiplying the number of antibiotic concentrations tested, it would be possible to determine precise MIC values for bacterial pellets obtained from blood culture, as previously shown from agar-grown bacteria [2]. Indeed, MIC is important for managing specific infections (e.g. *Streptococcus pneumoniae* and β-lactams).

Our highly encouraging results demonstrate the feasibility of the nanomotion-AST system coupled to a blood culture pellet, and efforts are under way to develop and accommodate this technique for routine diagnostic clinical laboratories. Future developments and studies might address the application of nanomotion-AST on bacteria recovered directly from samples if the bacterial load is sufficient or in combination with methods for the recovery of low numbers of bacteria in paucibacillary clinical samples. Such a procedure would greatly affect medical practice by changing the methodologic approaches currently in use to face the health threats posed by bacterial infections, particularly those arising from increasing multidrug resistance.

Acknowledgements

We thank C. Benadiba for her help in setting up the nanomotion-AST experiments, and C. Durussel and S. Gizha for technical assistance with the bacteriologic part of the experiment.

Transparency declaration

PS, GL, SK and GD were funded by Swiss National grant 200021-144321. SK and GD acknowledge the support of Gebert Rüf Stiftung GRS-024/14 and Centre Cooperation & Developement Program Seed Money 2016. GL acknowledges the support of Italian Health Ministry grant GR-2009-1605007. All authors report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2016.12.028.

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