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ORIGINAL ARTICLE

Digital antimicrobial susceptibility testing using the MilliDrop technology

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Abstract We present the MilliDrop Analyzer (MDA), a droplet-based millifluidic system for digital antimicrobial susceptibility testing (D-AST), which enables us to determine minimum inhibitory concentrations (MICs) precisely and accurately. The MilliDrop technology was validated by using resazurin for fluorescence readout, for comparison with standard methodology, and for conducting reproducibility studies. In this first assessment, the susceptibility of a reference Gram-negative strain *Escherichia coli* ATCC 25922 to gentamicin, chloramphenicol, and nalidixic acid were tested by the MDA, VITEK®2, and broth microdilution as a reference standard. We measured the susceptibility of clinically relevant Gram-positive strains of *Staphylococcus aureus* to vancomycin, including vancomycin-intermediate *S. aureus* (VISA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA), and vancomycin-susceptible *S. aureus* (VSSA) strains. The MDA provided results which were much more accurate than those of VITEK®2 and standard broth microdilution. The enhanced accuracy enabled us to reliably discriminate between VSSA and hVISA strains.

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Introduction

An important task of the clinical microbiology laboratory is to perform antimicrobial susceptibility testing (AST), which is essential for guiding the treatment of bacterial infections, especially in the current context of increasing rates of antibiotic resistance [1, 2]. At present, AST is typically accomplished using either conventional manual methods, including broth microdilution and disk diffusion, or an automated system, such as the VITEK®2 (bioMérieux), the MicroScan WalkAway (Beckman Coulter), and Phoenix (Becton Dickinson) [3–8]. However, all these methods have in common that they require a relatively large number of viable microorganisms and, usually, the antibiotic concentration gradients are not very accurate. For example, the antibiotic concentration gradient is formed by two-fold serial dilution in broth microdilution, which is recommended as the gold standard by the Clinical and Laboratory Standards Institute (CLSI) [9]. Consequently, any strain with an actual minimum inhibitory concentration (MIC) between 1 and 2 mg/L would be assigned a value of 2 mg/L. Moreover, this method is known to be reproducible up to one two-fold dilution [10]. A strain with an MIC of 2 mg/L could give rise to values of 1 or 4 mg/L, hence a two-fold dilution difference. This lack of precision can be detrimental in cases when MIC values are close to the clinical breakpoints or when it is needed to detect decreased susceptibility or heterogeneous resistance. This is exactly the case for the susceptibility testing of *Staphylococcus aureus*, a major cause of hospital- and community-associated infections worldwide, to vancomycin [11, 12].

Droplet-based technology has emerged as a powerful platform for performing chemical and biological experiments, with the advantage of high throughput, low sample consumption, and minimum cross-contamination in a virtually closed system [13–17]. Benefiting from these features, droplet-based

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technology has proven its ability to support experiments with DNA, protein, cells, and organisms to address different biological challenges [18–22]. Thousands of droplets can be generated in seconds to minutes. Each droplet can host an individual biochemical reaction, allowing thousands of reactions to be performed in minutes with small amounts of reagent [23]. Several droplet-based microfluidic AST devices have been studied, showing that this technology holds great potential to supplant the existing toolbox of microbiologists [24–28]. For example, the detection of bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics has been demonstrated [29]. Though it is technically possible, no formal comparison has yet been made between droplets-based AST technology and standard AST methods to justify their use in routine clinical practice.

Here, we developed a droplet-based millifluidic system, the MilliDrop Analyzer (MDA), for digital antimicrobial susceptibility testing (D-AST) that allows the growth of single to million bacterial cells in nanoliter droplets. The feasibility of this technology has previously been established in our lab for AST measurements on fluorescently labeled *Escherichia coli* [24]. In the present work, we optimized the performance of the MilliDrop system to provide reproducible and accurate AST on non-fluorescent clinical strains. Secondly, the performance of the Millidrop system was compared with those of standard broth microdilution, as well as VITEK®2 and Etest® technologies.

Materials and methods

Bacterial strains

The reference strains *E. coli* ATCC 25922, vancomycin-intermediate *S. aureus* (VISA) ATCC 700699, heterogeneous vancomycin-intermediate *S. aureus* (hVISA) ATCC 700698, and vancomycin-susceptible *S. aureus* (VSSA) ATCC 29213 were used. All the strains were kept at −80 °C in nutritive broth plus 15 % glycerol (bioMérieux, France). Before experiments, the isolates were subcultured overnight on Columbia agar with 5 % sheep blood at 35 °C prior to testing. The *S. aureus* VISA and hVISA were grown overnight at 35 °C in BHI agar (Difco BD, France), containing 2 mg/L of vancomycin.

Antibiotics

Gentamicin, chloramphenicol, nalidixic acid, and vancomycin were provided by bioMérieux. All of the antibiotic stock solutions were prepared according to the CLSI guidelines (M100-S23, January 2013). The antibiotic concentration ranges that were tested are reported in Table 1.

Table 1 Antibiotics and antimicrobial concentration ranges tested

Antibiotic	Concentration range	Species
Gentamicin	0.031–16 mg/L	*Escherichia coli*
Chloramphenicol	0.125–64 mg/L	
Nalidixic acid	0.125–64 mg/L	
Vancomycin	0.125–64 mg/L	*Staphylococcus aureus*

Broth microdilution

Broth microdilution assays were performed in 96-well microplates with two-fold dilutions of antibiotics prepared prior to bacterial inoculation. A single colony cultured from an agar plate was suspended in 0.85 % NaCl to reach a concentration of 0.5 McFarland and then diluted by 1/100 in cation-adjusted Mueller Hinton Broth (Difco BD, France), as recommended by the CLSI (M100-S23, January 2013). The MICs were determined by visual reading after 20 h of incubation at 35 °C for *E. coli* and after 24 h of incubation for *S. aureus*.

VITEK®2

For susceptibility testing by VITEK®2, AST-N233 and AST-P631 cards were used for *E. coli* and *S. aureus* respectively. Bacterial suspensions were prepared in 0.45 % saline to obtain a 0.5 McFarland turbidity and then diluted to 0.054 (*S. aureus*) or 0.025 McFarland (*E. coli*), which correspond respectively to approximately 1.6×10^7 or 7.5×10^6 colony-forming units (CFU)/mL, as recommended by the manufacturer.

Etest®

Etest® susceptibility testing was performed according to the manufacturer's guidelines.

MDA design and operation

We have previously reported the use of a millifluidic droplet analyzer for precisely monitoring the dynamics of microbial populations in aqueous emulsion droplets [24]. For the present work, we adapted this Millidrop technology to D-AST using resazurin as a fluorescent biomass reporter, which allows the analysis of a broader range of species. The schematic diagram of the MDA is described in Fig. 1. Liquid medium (Mueller Hinton Broth, bioMérieux, France), antibiotic solution, and bacterial suspension are converged at the first junction into an aqueous stream, which then meets the carrier fluid (HFE oil, Novec, France) and the spacing fluid (mineral oil, Sigma-Aldrich, France) at the second junction. HFE oil, as an immiscible phase, is injected at a constant flow rate to break up the aqueous stream (bacteria and antibiotic

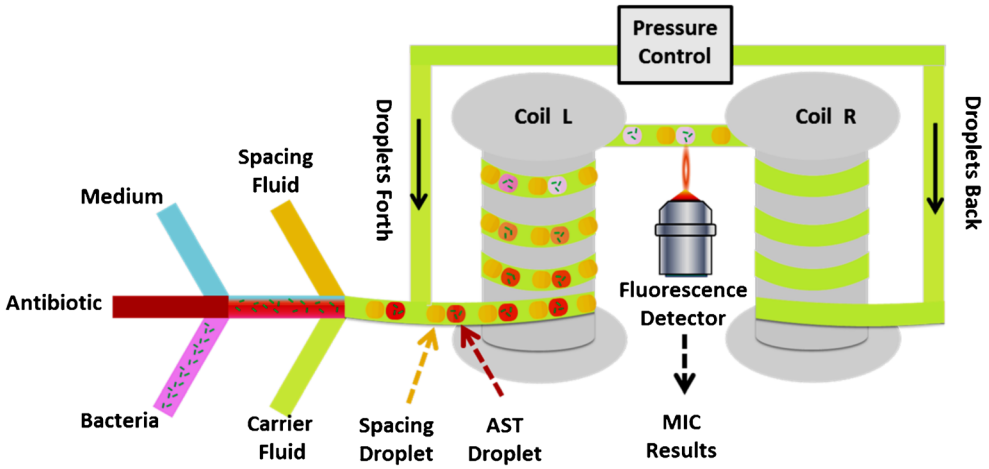

Fig. 1 Schematic of the MilliDrop Analyzer (MDA). Liquid medium, antibiotic containing sulforhodamine as gradient reporter, and bacterial suspension with resazurin as biomass reporter are converged at the first junction into an aqueous stream. At the second junction, carrier fluid (HFE oil) breaks this aqueous stream and spacing fluid (mineral oil) into alternating antimicrobial susceptibility testing (AST) droplets with defined composition (bacteria plus antibiotic mixture) and spacing droplets (mineral oil). The droplet sequence is driven back and forth between tubing coil L and coil R during the incubation period by a pressure controller. When droplets passed through the fluorescence detector, the biomass and antibiotic concentration in each AST droplet are measured by independent fluorescent signals. The minimum inhibitory concentration (MIC) can be determinated by plotting the growth profiles in AST droplets

mixture) and the spacing fluid into alternating AST droplets (aqueous droplet) and spacing droplets (mineral oil droplet) with constant volume. Each AST droplet functions as an individual bioreactor with spacing droplets, allowing for spatial separation of the actual AST droplets.

In each test, an aqueous stream with a constant bacterial density of 1.5×10^6 CFU/mL and altering antibiotic concentration in cation-adjusted Mueller Hinton Broth was injected to generate an AST droplet sequence (about 250 bacteria/droplet). This inoculum size was determined in a preliminary study (results not shown). Antibiotic concentration gradients across the AST droplet sequences was achieved by adjusting the flow rates of liquid medium and antibiotic solution with a three-step injection process. In the first step, the flow rates of liquid medium and antibiotic solution are kept constant at 25 μL/min and 1 μL/min, respectively, for 1 min. In the second step, the flow rate of liquid medium is decreased linearly from 25 μL/min to 1 μL/min in 2 min, while the flow rate of antibiotic solution is increased linearly from 1 μL/min to 25 μL/min. In the third step, the flow rates of liquid medium and antibiotic solution are kept constant at 1 μL/min and 25 μL/min, respectively, for 1 min. The flow rate of bacterial suspension is kept constant at 7 μL/min during the whole three-step injection process. The sum of the three flow rates remains constant during droplet generation. A total sample volume of 132 μL is injected and divided into a droplet sequence consisting of approximately 800 ± 25 AST droplets and an equal number of spacing droplets in 4 min.

The droplet sequence was carried by HFE oil into a 7-m transparent capillary FEP tube (0.5 mm inner diameter, IDEX, USA), which was arranged into two coils (coil L and coil R) with equal length and incubated at 35 ± 1 °C. During the test, the droplet sequence was moved back and forth regularly by a pressure controller in front of a fluorescence detector, which was placed in the middle of the two coils. To quantify the final concentration of antibiotic in each AST droplet, 1 mM of red fluorescent sulforhodamine 101 (Life Technologies, USA) was premixed with the antibiotic solution. Likewise, 87 μM of resazurin (Sigma-Aldrich, USA) was premixed with the bacterial suspension to act as a metabolic reporter of bacterial growth in the droplets [25, 29]. Blue and non-fluorescent resazurin is irreversibly reduced into pink and highly fluorescent resorufin by viable bacteria.

The biomass and antibiotic concentration in each AST droplet were tracked by independent fluorescent signals when droplets passed through the detector. MDA operations and data acquisition were achieved by a custom-made LabVIEW (National Instruments, USA) application. In each AST droplet, the sulforhodamine signal (proportional to the antibiotic concentration) and the resorufin signal (proportional to the number of viable cells) were recorded every 10 min over a period of 6–18 h, depending on the bacteria/antibiotic combinations. Data analysis was performed by a custom-made R application.

Algorithm-based MIC estimation

In D-AST, the first data set obtained was the antibiotic concentration in each AST droplet (sulforhodamine signal), which was acquired in the first run when all the droplets pass through the detector (shown in Fig. 2a). The second data set

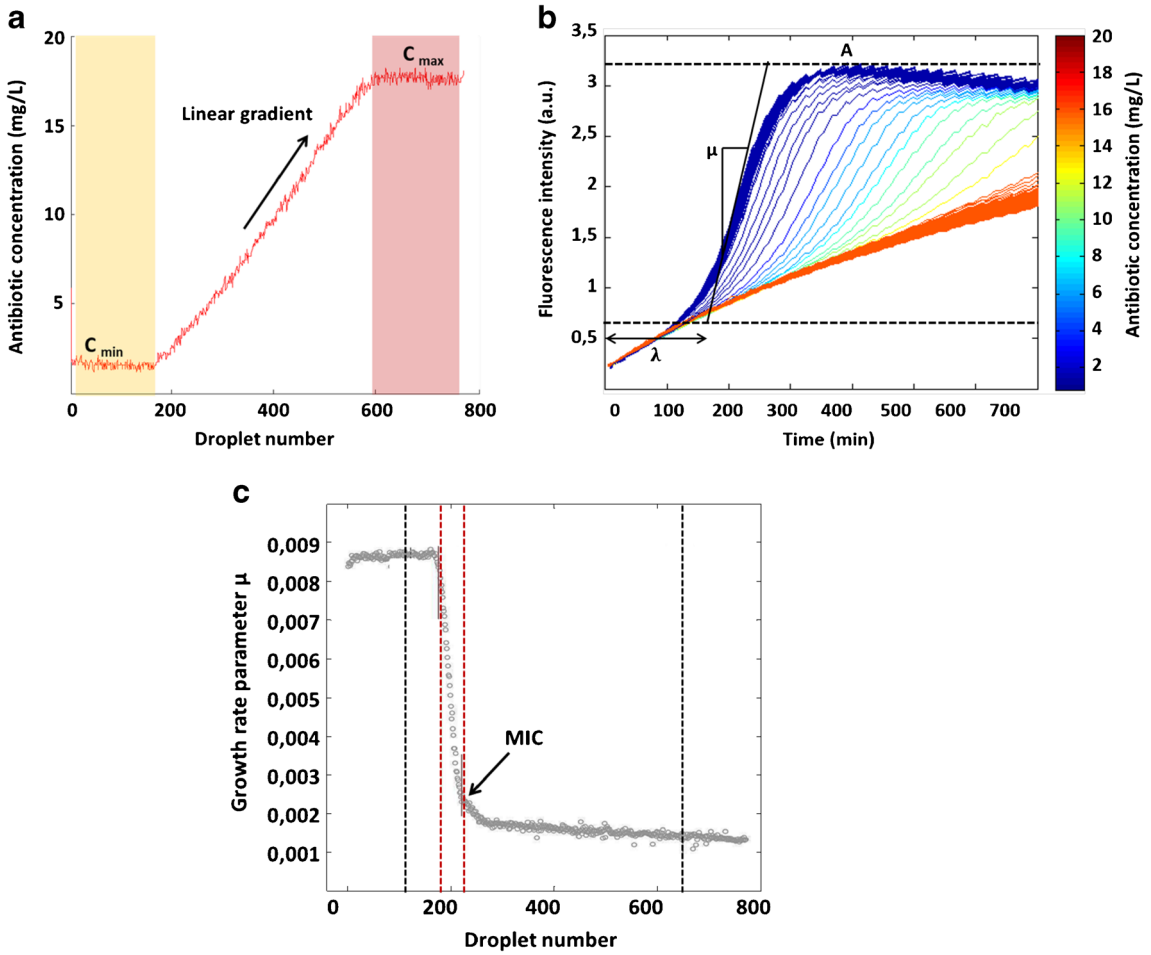

Fig. 2 MIC measurement in the MDA. **a** Concentration gradient in the AST droplet sequence for the testing of chloramphenicol/*E. coli*. **b** Typical growth curves of chloramphenicol against *E. coli* in the droplet sequence (1/5 of the full sequence is plotted here). Each curve represents the growth profile in a single droplet. The growth profile of the first droplet is characterized by the length of the lag phase (λ), maximum growth rate (μ), and maximum biomass. **c** MIC estimation by calculating the growth rate parameter μ after 6 h of incubation

was the growth profile (resorufin signal) associated to each AST droplet, which was logged as a vector of numerical fluorescence values over time when pressure drove the droplet train back and forth in front of the detector (shown in Fig. 2b). These data sets were then used to determine the MIC by analyzing the growth profile in AST droplets along the antibiotic concentrations in the train.

To derive conclusive concentration–response growth curves for MIC estimation, we adapted an algorithm previously used for fitting growth curves obtained under different conditions [30, 31], as illustrated in Fig. 2b. The relationship between biomass and the bacteria growth rate could be described by the following equation:

$$y(t) = A.exp\left\{-exp\left[\frac{\mu.e}{A}(\lambda - t) + 1\right]\right\}$$

where y presents the biomass at stationary phase in each droplet, A is the maximum bacterial growth, λ is the duration of the lag phase, and μ is the growth rate at exponential phase. As an alternative to the visual determination of the MIC, μ was exploited to phenotypically discern the antibiotic susceptibility profile. μ at different concentrations of chloramphenicol against the reference *E. coli* strain was determined by applying this algorithm. μ values at a predefined time (time to reach stationary phase in the first droplet) plotted on the corresponding serial number of a droplet is shown in Fig. 2c. The black, dashed vertical lines correspond to the minimum and maximum antibiotic concentrations, respectively, as described previously. Droplets that presented a maximum μ value have much lower antibiotic concentrations than the MIC, within which the bacteria growth is, therefore, unperturbed. Conversely, droplets that showed a minimum μ value have higher concentrations than the MIC, within which the growth was, therefore, inhibited. Droplets in between were related to the sub-MIC range represented by the vertical red lines. In the present study, we considered the lowest concentration with a minimum μ value as the MIC of the strain in the assay.

Results

Method evaluation

To evaluate the performance of the MDA for AST, gentamicin, chloramphenicol, and nalidixic acid were tested against the reference *E. coli* strain ATCC 25922. Parallel experiments were carried out as well with VITEK®2 and broth microdilution to investigate any discordance between the MDA and standard methods. To study the reproducibility of the MDA, each experiment was performed in five independent replicates. Results obtained by using the algorithm-based MIC estimation method after 6 h of incubation are summarized in Table 2. We noted that 13 results out of 15 provided by the MDA were concordant with the quality control MIC ranges provided by the CLSI, whereas two chloramphenicol-based assays reported lower MICs than the quality control range. The MIC results reported by broth microdilution and VITEK®2 were different in both the tests of chloramphenicol/*E. coli* and nalidixic acid/*E. coli*; however, these results were still within the quality control ranges. According to the MIC results of each quintuple assay, the coefficients of variation (CV) were 18.2 %, 31.6 %, and 34.8 % for nalidixic acid/*E. coli*, gentamicin/*E. coli*, and chloramphenicol/*E. coli*, respectively.

VISA and hVISA discrimination with the MDA

We conducted further tests with the MDA and vancomycin, a clinically relevant drug, by testing three reference strains of *S. aureus* with a known resistance phenotype. Each assay was performed in five replicates and the results are presented in Table 3. All the results reported by broth microdilution and VITEK®2 were in concordance with the expected MIC ranges. The MDA MIC results of the VSSA strain were all in the expected MIC ranges, whereas the MICs of the hVISA and VISA strains were below the expected MIC ranges but still consistent with the CLSI norms. The standard deviation was as low as 18 %. Moreover, VSSA, hVISA, and VISA strains can be discriminated through the MIC results in the MDA, whereas broth microdilution cannot distinguish between VSSA and hVISA, and VITEK®2 is not always able to discriminate between hVISA and VSSA or between VISA and hVISA. As for the MDA, the Etest® allowed the discrimination through the MIC values, but the MICs were sometimes higher than those of the expected MIC ranges.

Discussion

In this work, the MDA was developed for D-AST and a new algorithm was adapted to better estimate MIC values. The comparison was made with the commonly used automated system VITEK® 2, Etest®, and with standard broth microdilution.

For the most part of the reproducibility study, the MIC results of the MDA were consistent and comparable with those of standard methods. Two results out of 15 were slightly below the reference MIC range. These outliers can be explained by mild flow rate disturbances during the droplet generation, which may cause an underestimation of the minimum antibiotic concentration (Cm), further resulting in MIC underestimates (SWOT analysis of the MDA is shown in Table S1). To address this issue, we improved the MDA to guarantee stable and smooth flow rates during the formation of droplet sequences.

Table 2 Comparison of minimum inhibitory concentrations (MICs) assessed by broth microdilution, VITEK®2, and the MilliDrop Analyzer (MDA)

Antibiotic/strain	MIC (mg/L)				CV	Concordance rate
	Quality control ranges	Broth microdilution	VITEK®2	MDA		
Gentamicin/*E. coli*	0.25–1	0.5	0.5	0.64 0.46 0.40 0.26 0.41	31.6 %	5/5
Chloramphenicol/*E. coli*	2–8	2	4	2.04 2.40 0.80 2.17 1.63	34.8 %	3/5
Nalidixic acid/*E. coli*	1–4	2	≤2	1.09 1.52 1.83 1.40 1.58	18.2 %	5/5

Table 3 Discrimination of vancomycin-susceptible *S. aureus* (VSSA), vancomycin-intermediate *S. aureus* (VISA), and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) by broth microdilution, VITEK®2, Etest®, and the MDA

Strains	MIC (mg/L)				
	Quality control ranges	Broth microdilution	VITEK®2	Etest®	MDA
VSSA ATCC 29213	0.25–1	0.5–1	0.5–1	1.5	0.40
					0.42
					0.46
					0.51
					0.59
hVISA ATCC 700698	1–4	1–2	1–4	3–6	0.86
					0.99
					0.99
					1.15
					1.22
VISA ATCC 700699	4–8	4–8	4	8–12	1.79
					2.42
					2.52
					2.89
					3.54

In current methodologies for MIC breakpoint determination, result accuracy is extremely important when characterizing new antimicrobial compounds against different bacteria or when designing the optimal antimicrobial therapy, especially in case of severe infections [32]. Accurate MICs could be valuable for choosing the most appropriate drugs and regimens, especially when MICs are close to the clinical breakpoints, which could change clinical categorizations [33]. Currently, the Etest® is more often used when clinicians need more precise results than what automated or broth microdilution tests provide. In this study, we demonstrated that the MDA could deliver more accurate and precise MIC results than Etest®, VITEK® 2, and broth microdilution, owing to the large number of droplets (800 droplets containing increasing concentrations of antibiotics).

The MDA and the other three methods were employed to discriminate VSSA, VISA, and hVISA strains here. The MDA was shown to be able to discriminate clearly these three strains, whereas the MIC intervals overlapped in both VITEK®2 and broth microdilution. The Etest® also allowed the discrimination, although the values were not always in the expected ranges.

A particular advantage of the MDA compared to conventional AST techniques is that the large number of droplets provides a unique tool for rapid phenotypic bacterial screening. This enabled the discrimination of resistant strains from intermediate variants through the growth in droplets (shown in supplementary material Fig. S1). Without the large number of individual AST droplets, the heterogeneity may have been overlooked. The distinction of resistant mutants can be conducted by the population analysis profiling (PAP) method as well. However, the PAP method is fastidious, takes a long time to develop, and is not well standardized, whereas the MDA uses mathematical algorithms, which provide a more objective readout. Furthermore, the ability of the MDA to sort and collect droplets could allow for further characterization and understanding of related heteroresistance mechanisms.

Another important AST aspect is the time to result, which should be as short as possible to provide clinicians with early AST results. The minimum incubation time to get MIC values has been shortened in the MDA compared to standard AST methods, for which at least 16 h is often required. In the MDA, reliable MIC results can be obtained in less than 2 h when we test fast-growing strains such as *E. coli*. Nevertheless, even without antibiotics, bacteria have a lag period before starting to grow exponentially [34, 35]. This biologically defined timespan cannot be shortened at present. However, by decreasing the assay time too much, one will underestimate the MIC. Hence, there is a tradeoff between fast MIC results and accurate values.

Before proceeding to clinical implementations, further developments will be conducted to extend the applications of the MDA for clinical routine AST. Firstly, future design will be adapted to test multiple antibiotics by incubating several independent droplet sequences in one assay. Secondly, the sensitivity of the laser signal needed to detect biomass directly by optical density without using any fluorescent reporter must be improved. Finally, more clinical isolates of both Gram-negative and Gram-positive origin must be tested by the MDA. We anticipate that the MilliDrop technology could be broadly applied to test clinical antimicrobial susceptibility in a large variety of bacterial infections. We also expect that the MilliDrop technology will serve as a useful tool in fundamental microbiology in the near future.

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Compliance with ethical standards

Conflict of interest Patrick Broyer, Anne-Coline Chareire, Pierrot Bourne-Branchu, Pierre Mahé, Maud Tournoud, Christine Franceschi, and Gilles Zambardi are scientists employed by bioMérieux. Laurent Boitard, Jean Baudry, and Jérôme Bibette are founders of MilliDrop Instruments SAS. Lianmei Jiang declares no conflict of interest.

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