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ORIGINAL ARTICLE

Dry Plate-Based Direct-On-Target Microdroplet Growth Assay for Rapid Antimicrobial Susceptibility Testing of MRSA

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ABSTRACT

Background: **Rapid antimicrobial susceptibility testing (AST) is crucial for the early optimization of treatment against infections caused by drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). Direct-on-target microdroplet growth assay (DOT-MGA), which uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has emerged as a promising phenotypic method for rapid AST. The present study aimed to evaluate the feasibility of DOT-MGA for resistant isolates, including the optimization of test conditions.**

Methods: **The feasibility and performance of DOT-MGA were evaluated using a commercial dry plate (DP42) for the AST of MRSA strains, including a clinical isolate with low susceptibility to daptomycin (ATCC 29213, ATCC 43300, and KAM784). DOT-MGA was performed under varying conditions (standard or 10-fold inoculum; 6/8-hour incubation). The minimum inhibitory concentrations (MICs) were compared with those from the reference DP42 method based on their agreement and deviation.**

Results: **DOT-MGA successfully estimated MICs within 6 - 8 hours. With the standard inoculum, the assay exhibited high concordance with the DP42 results, particularly for the ATCC strains. In contrast, a 10-fold increase in inoculum led to increased MIC values, consistent with the inoculum effect. The daptomycin-low-susceptibility strain (KAM784) exhibited decreased MICs in standard inoculum and short incubation periods, suggesting a risk of under detection in resistant strains. MIC agreement was influenced by both the antibiotic type and MIC relative to the DP42 test range.**

Conclusions: **DOT-MGA is a rapid phenotypic AST method for MRSA that can be implemented using MALDI-TOF MS and commercial reagents. Optimization of the inoculum size and incubation time is necessary to ensure accuracy, particularly for detecting reduced susceptibility to key antibiotics such as daptomycin and linezolid.**
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KEYWORDS

MRSA, direct-on-target microdroplet growth assay, daptomycin, rapid antimicrobial susceptibility testing, MALDI-TOF MS

INTRODUCTION

The global spread of antimicrobial resistance (AMR) is a serious concern that urgently demands rapid and accurate antimicrobial susceptibility testing (AST) in clinical practice [1]. Conventional culture-based AST requires at least 18 hours of processing, during which the

use of empiric broad-spectrum antibiotics is often unavoidable. However, early initiation of appropriate antibiotic therapy is critical for improving patient outcomes [2,3]. Rapid same-day AST is essential to promptly adjust antimicrobial regimens, improve laboratory workflow, and reduce healthcare costs [4]. Thus, the acceleration of AST has become an important priority in clinical microbiology and the development of global AMR countermeasures and antimicrobial stewardship strategies [5].

Advancements in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have dramatically reduced the time required for bacterial identification, while rapid AST development has lagged [6]. Among recent innovations, direct-on-target microdroplet growth assay (DOT-MGA) using MALDI-TOF MS has gained attention as a promising rapid AST technique [7]. DOT-MGA involves culturing bacteria in microdroplets of medium (a few microliters) on a MALDI target plate with antibiotics, followed by MALDI-TOF MS analysis after a short incubation period. To prevent evaporation, the droplets are incubated in a humidified chamber, after which the residual fluid is removed with absorbent paper, and the samples are processed for MALDI analysis. The presence of bacterial growth is determined based on the MALDI Biotyper score: a value of ≥ 1.7 indicates resistance, whereas a lower score suggests susceptibility due to growth inhibition. A key advantage of DOT-MGA is its ability to simultaneously identify bacteria and their susceptibility from the same culture spot. This property enables both rapid diagnostics and the detection of possible contaminant species, which would otherwise be indistinguishable using traditional methods. In addition, this method is simple to perform using commercially available reagents and media, does not require specialized consumables, and can detect phenotypic resistance mechanisms that may be missed by genetic assays. Unlike commercial dry plate systems, DOT-MGA allows for simultaneous evaluation of multiple antibiotics and strains on a single MALDI target plate [8]. Collectively, these features render DOT-MGA a highly attractive solution for same-day AST using widely available MALDI-TOF MS systems.

Since 2018, an increasing number of studies have applied DOT-MGA, primarily to Gram-negative bacteria, for rapid AST development [9]. Idelevich et al. [7] demonstrated that the carbapenem susceptibility of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* can be determined within 4 hours by culturing meropenem-containing droplets directly on MALDI targets. Moreover, DOT-MGA has been adapted for direct meropenem susceptibility testing in blood culture specimens, with high sensitivity and specificity after 4 hours of processing [10]. Panel-based assays using DOT-MGA have also been developed for detecting resistance mechanisms such as extended-spectrum β-lactamases, AmpC, and carbapenemases, achieving accuracies comparable to polymerase chain reaction within 4 hours [11,12]. However, the application of DOT-MGA to Gram-positive bacteria remains limited. The earliest report described its use in methicillin-resistant *Staphylococcus aureus* (MRSA) tested using cefoxitin-DOT-MGA, demonstrating high accuracy within a 4-hour incubation period [13]. However, limited data exist for vancomycin, linezolid, and daptomycin, which are critical drugs for the treatment of MRSA, highlighting the need for determining their minimum inhibitory concentrations (MICs), especially by using rapid phenotypic methods. Overall, DOT-MGA is a practical approach for rapid phenotypic AST that leverages the widespread availability of MALDI-TOF MS systems. Although other technologies, such as genotypic assays and microfluidic platforms, are also under development, DOT-MGA is one of the most feasible options for routine clinical implementation owing to its versatility, ease of use, and cost-effectiveness. Although ongoing research continues to expand its utility, further data accumulation and methodological standardization are needed to support broader adoption [9]. Therefore, in this study, we explored the applicability of DOT-MGA to MRSA strains with reduced susceptibility to daptomycin, a clinically important yet underexplored area. Specifically, we aimed to evaluate the feasibility of DOT-MGA for these resistant isolates, optimize test conditions, including bacterial inoculum and incubation time, and compare the performance across multiple antibiotics.

MATERIALS AND METHODS

Bacterial strains

Three *S. aureus* strains were used in this study: ATCC 29213 was used as the reference quality-control (QC) strain for AST; ATCC 43300, a standard MRSA strain, was used as the resistant control; and KAM784, a clinical MRSA isolate with low susceptibility to daptomycin, which was provided by Kitasato University Hospital (Kanagawa, Japan). The MIC of daptomycin was 2 μg/mL in KAM784, as determined by the DP42 method.

Pre-culture and inoculum preparation

All strains were subcultured on trypticase soy agar (Eiken Chemical, Tokyo, Japan) and incubated at 35°C for 24 hours. Colonies were suspended in sterile saline and adjusted to an optical density of 0.3 at 590 nm. A 50-μL aliquot of this suspension was added to 12 mL Mueller-Hinton broth (Eiken Chemical) to a final concentration of 5 x 10^4 colony-forming unit (CFU)/mL.

AST using DP42

MICs were determined using a dry plate DP42 system (Eiken Chemical) with predefined antimicrobial concentrations. The prepared bacterial suspensions were dispensed into each well and incubated at 35°C for 18 hours. MICs were visually determined as the lowest antimicrobial concentration that completely inhibited visi-

ble bacterial growth. The antimicrobials, concentration ranges, and ATCC 29213 QC ranges are summarized in Table 1.

Direct-on-target microdroplet growth assay (DOT-MGA)

DOT-MGA was performed as previously described, with minor modifications [13]. Briefly, bacterial suspensions were inoculated onto each well of the DP42 plate, and a 6-μL aliquot of the mixture was spotted on disposable target plates for a MALDI Biotyper Sirius platform (Bruker Daltonik GmbH, Bremen, Germany). Spotted plates were incubated at 35°C in a humidified chamber for 6 or 8 hours. After incubation, the supernatants were removed using square filter paper No. 1 (Kenis, Osaka, Japan), and the plates were air-dried at room temperature. Each spot was overlaid with 1 μL of 70% formic acid, followed by 1 μL IVD Matrix HCCA (Bruker Daltonik). After drying, spectra were acquired using the MALDI Biotyper Sirius (Bruker Daltonik). Spots with identification scores ≥ 1.7 were defined as positive for bacterial growth. MICs were determined based on growth/no-growth patterns across antimicrobial concentrations. The assay was conducted under four conditions: standard and high-inoculum size (x 1 and x 10) and two incubation periods (6 hours and 8 hours). Two independent experiments were performed under each condition.

RESULTS

MICs determined using DP42

The DP42 dry plate method revealed that the MICs of the QC strain ATCC 29213 (KAM61) were within the CLSI-defined QC ranges for all antibiotics tested. Both ATCC 43300 (KAM246) and KAM784 exhibited high MICs for oxacillin and cefoxitin, confirming their identities as MRSA strains.

MICs determined using DOT-MGA

We compared the MICs obtained using the DOT-MGA method under different conditions (inoculum x 1/x 10, incubation 6/8 hours) with the DP42 results; the differences (termed “tube difference”) are visualized in Figure 1. Overall, using 10 x inoculum tended to yield higher MICs than using DP42. In contrast, under standard inoculum (x 1) conditions, ATCC 29213 and ATCC 43300 exhibited high concordance and reproducibility. In KAM784 cells, MICs were generally underestimated under standard inoculum conditions. We observed no clear trend between the 6-hour and 8-hour incubation times.

Agreement with DP42 under each culture condition

We assessed the agreement between DOT-MGA and DP42 under each condition (inoculum: x 1/x 10, incubation time: 6/8 hours) for each strain (Figure 2). The MICs of all strains tended to be overestimated under conditions of x 10 inoculum. ATCC 29213 and ATCC 43300 demonstrated minimal (≥ 2-fold) discrepancies under x 1 inoculum, indicating the reproducibility of the DOT-MGA method. In contrast, the MIC of KAM784 was underestimated with the standard inoculum. We observed no notable effect in terms of incubation time.

Antibiotic-wise agreement

We assessed the agreement between DOT-MGA and DP42 using different antibiotics and categorized them based on inoculum size (x 1 vs. x 10; Figure 3). Under x 10 inoculum, multiple antibiotics, including oxacillin, penicillin G, daptomycin, levofloxacin, and sulfamethoxazole/trimethoprim, exhibited high MIC values, resulting in reduced concordance. In contrast, fosfomycin, gentamicin, and cefoxitin demonstrated high agreement between methods regardless of inoculum size.

Agreement stratified by MIC ranges relative to DP42 detection limits

We compared the DOT-MGA and DP42 MIC results by stratifying the target strain MICs into three categories: below, within, or above the DP42 detection range (Figure 4). With the x 1 inoculum, we noted high concordance between the methods for strains with MICs below the DP42 range. However, MICs were frequently overestimated with the x 10 inoculum. Notably, the strains with MICs above the DP42 range tended to exhibit better concordance under conditions of x 10 inoculum.

DISCUSSION

Considering the global spread of antimicrobial-resistant organisms and pressing need for antimicrobial stewardship, the demand for rapid AST is increasing annually. This is especially critical in cases of sepsis or severe infections, where even a 1-day delay in diagnosis can greatly affect prognosis [14]. Numerous next-generation rapid AST technologies have been developed in recent years. Among these, phenotypic AST can directly assess antimicrobial effects within a short time, without relying on the detection of resistance genes, enabling the detection of both known and novel resistance mechanisms [15,16]. Importantly, phenotypic AST can identify discrepancies between genotypes and phenotypes. These include cases in which resistance genes are present but not expressed or where resistance is phenotypically evident despite the absence of detectable resistance genes, making phenotypic AST highly suitable for adaptive clinical management. However, many newly developed rapid AST platforms require expensive cartridges and dedicated instruments. Moreover, their widespread implementation is hindered by high costs and operational complexity [17]. In contrast, DOT-MGA leverages commercially available materials and existing MALDI-TOF MS equipment, providing a cost-effective, scalable, and user-friendly method for clinical laboratories. Therefore, in the present study, we evalu-

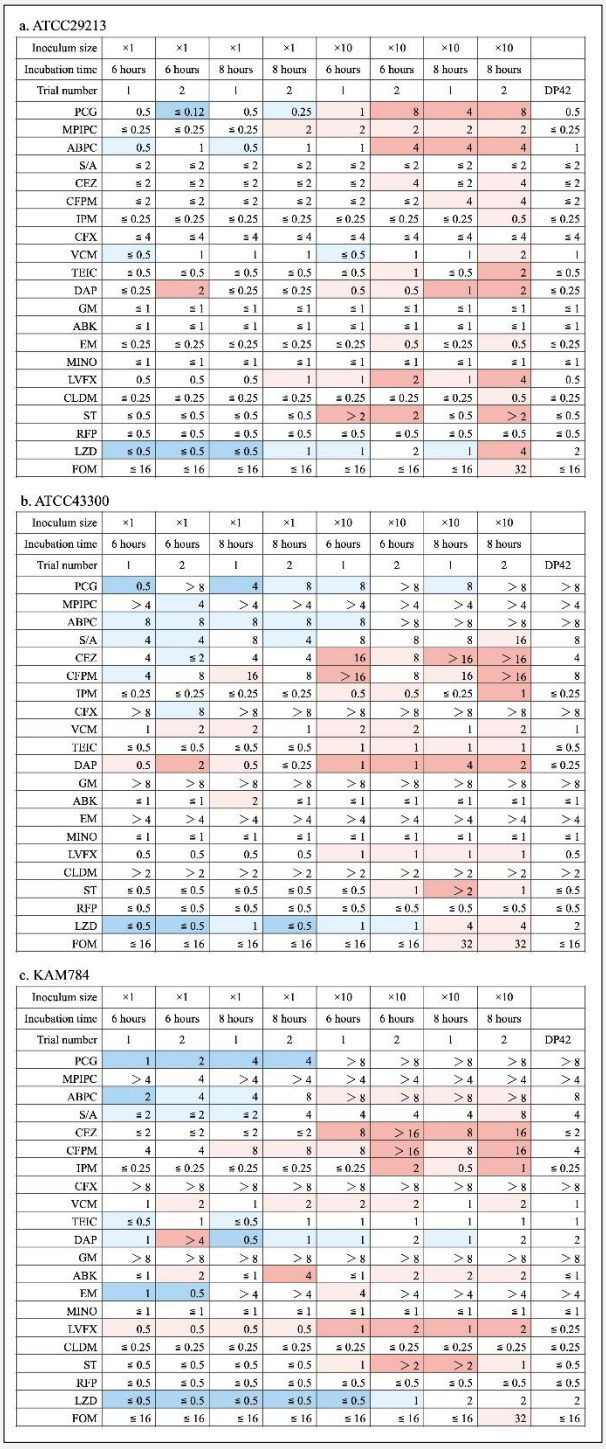
Table 1. MIC ranges of DP42, QC limit, and MICs of tested strains.

	DP42 range		QC range		MICs based on DP42		
	Lower	Upper	Lower	Upper	ATCC 29213	ATCC 43300	KAM 784
PCG	0.12	8	0.25	2	0.5	> 8	> 8
MPIPC	0.25	4	0.12	0.5	≤ 0.25	> 4	> 4
ABPC	0.25	8	0.5	2	1	> 8	8
S/A	2	16			≤ 2	8	4
CEZ	2	16	0.25	1	≤ 2	4	≤ 2
CFPM	2	16	1	4	≤ 2	8	4
IPM	0.25	8	0.016	0.06	≤ 0.25	≤ 0.25	≤ 0.25
CFX	4	8	1	4	≤ 4	> 8	> 8
VCM	0.5	16	0.5	2	1	1	1
TEIC	0.5	16	0.25	1	≤ 0.5	≤ 0.5	1
DAP	0.25	4	0.12	1	≤ 0.25	≤ 0.25	2
GM	1	8	0.12	1	≤ 1	> 8	> 8
ABK	1	8	-	-	≤ 1	≤ 1	≤ 1
EM	0.25	4	0.25	1	≤ 0.25	> 4	> 4
MINO	1	8	0.06	0.5	≤ 1	≤ 1	≤ 1
LVFX	0.25	4	0.06	0.5	0.5	0.5	≤ 0.25
CLDM	0.25	2	0.06	0.25	≤ 0.25	> 2	≤ 0.25
ST	0.5	2	-	0.5	≤ 0.5	≤ 0.5	≤ 0.5
RFP	0.5	2	0.004	0.016	≤ 0.5	≤ 0.5	≤ 0.5
LZD	0.5	4	1	4	2	2	2
FOM	16	128	0.5	4	≤ 16	≤ 16	≤ 16

QC: range represents the limit of ATCC 29213 based on the CLSI statement M100-Ed35, PCG: penicillin G, MPIPC: oxacillin, ABPC: ampicillin, S/A: sulbactam/ampicillin, CEZ: cefazolin, CFPM: cefepime, IPM: imipenem, CFX: cefoxitin, VCM: vancomycin, TEIC: teicoplanin, DAP: daptomycin, GM: gentamicin, ABK: arbekacin, EM: erythromycin, MINO: minocycline, LVFX: levofloxacin, CLDM: clindamycin, ST: sulfamethoxazole/trimethoprim, RFP: rifampicin, LZD: linezolid, FOM: fosfomycin.

ated the feasibility and optimal conditions of DOT-MGA as a rapid and simplified method for the AST of MRSA.

Compared with conventional broth microdilution or Etest methods, DOT-MGA enables the estimation of MICs within 6 - 8 hours [10]. In this study, the use of commercial dry plates (DP42, Eiken Chemical) combined with a MALDI-TOF MS system eliminated the need for special reagents or costly instruments. Moreover, we investigated the optimal conditions for DOT-MGA, specifically the bacterial inoculum size and incubation time. Our results indicate that increasing the inoculum 10-fold to improve turnaround speed resulted in the frequent overestimation of MICs compared with those determined using DP42, consistent with previous findings [18]. This result is likely due to the inoculum effect, in which an increased bacterial load enhances resistance through mechanisms such as β-lactamase production. Some antibiotics, such as penicillin and cephalosporins, are more sensitive to inoculum size than others (e.g., carbapenems, fluoroquinolones) [18,19]. In our study, we noted pronounced inoculum effects with β-lactams, levofloxacin, and sulfamethoxazole/trimethoprim, which is partly consistent with previous findings. Conversely, although the MIC was underestimated for some β-lactams due to insufficient growth, the use of the standard inoculum (1 x) resulted in enhanced agreement with the DP42 results. Thus, a tradeoff exists between test speed and MIC accuracy and should be carefully considered in workflow optimization. In addition, the methods exhibited comparable MICs between the 6-hour and 8-hour conditions in our study. Turnaround times of 6 hours can promote rapid diagnosis and treatment. However, for KAM784, the MICs for erythromycin only matched those of DP42 after 8 hours. In general, this strain exhibited decreased MICs for the standard inoculum, possibly because of the slow growth rates typical of resistant strains. Collectively, our findings suggest that short incubation periods may not be suitable for screening slow-growing resistant organisms.



a. ATCC29213

Inoculum size	×1	×1	×1	×1	×10	×10	×10	×10	
Incubation time	6 hours	6 hours	8 hours	8 hours	6 hours	6 hours	8 hours	8 hours	
Trial number	1	2	1	2	1	2	1	2	DP42
PCG	0.5	≤ 0.12	0.5	0.25	1	8	4	8	0.5
MPIPC	≤ 0.25	≤ 0.25	≤ 0.25	2	2	2	2	2	≤ 0.25
ABPC	0.5	1	0.5	1	1	4	4	4	1
S/A	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
CEZ	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	4	≤ 2	4	≤ 2
CFPM	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	4	4	≤ 2
IPM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.5	≤ 0.25
CFX	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
VCM	≤ 0.5	1	1	1	≤ 0.5	1	1	2	1
TEIC	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	≤ 0.5	2	≤ 0.5
DAP	≤ 0.25	2	≤ 0.25	≤ 0.25	0.5	0.5	1	2	≤ 0.25
GM	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
ABK	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
EM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.5	≤ 0.25	0.5	≤ 0.25
MINO	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
LVFX	0.5	0.5	0.5	1	1	2	1	4	0.5
CLDM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.5	≤ 0.25
ST	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	> 2	2	≤ 0.5	> 2	≤ 0.5
RFP	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
LZD	≤ 0.5	≤ 0.5	≤ 0.5	1	1	2	1	4	2
FOM	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	32	≤ 16

b. ATCC43300

Inoculum size	×1	×1	×1	×1	×10	×10	×10	×10	
Incubation time	6 hours	6 hours	8 hours	8 hours	6 hours	6 hours	8 hours	8 hours	
Trial number	1	2	1	2	1	2	1	2	DP42
PCG	0.5	> 8	4	8	8	> 8	8	> 8	> 8
MPIPC	> 4	4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
ABPC	8	8	8	8	8	> 8	> 8	> 8	> 8
S/A	4	4	8	4	8	8	8	16	8
CEZ	4	≤ 2	4	4	16	8	> 16	> 16	4
CFPM	4	8	16	8	> 16	8	16	> 16	8
IPM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.5	0.5	≤ 0.25	1	≤ 0.25
CFX	> 8	8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
VCM	1	2	2	1	2	2	1	2	1
TEIC	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	1	1	1	≤ 0.5
DAP	0.5	2	0.5	≤ 0.25	1	1	4	2	≤ 0.25
GM	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
ABK	≤ 1	≤ 1	2	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
EM	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
MINO	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
LVFX	0.5	0.5	0.5	0.5	1	1	1	1	0.5
CLDM	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
ST	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	> 2	1	≤ 0.5
RFP	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
LZD	≤ 0.5	≤ 0.5	1	≤ 0.5	1	1	4	4	2
FOM	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	32	32	≤ 16

c. KAM784

Inoculum size	×1	×1	×1	×1	×10	×10	×10	×10	
Incubation time	6 hours	6 hours	8 hours	8 hours	6 hours	6 hours	8 hours	8 hours	
Trial number	1	2	1	2	1	2	1	2	DP42
PCG	1	2	4	4	> 8	> 8	> 8	> 8	> 8
MPIPC	> 4	4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
ABPC	2	4	4	8	> 8	> 8	> 8	> 8	8
S/A	≤ 2	≤ 2	≤ 2	4	4	4	4	8	4
CEZ	≤ 2	≤ 2	≤ 2	≤ 2	8	> 16	8	16	≤ 2
CFPM	4	4	8	8	8	> 16	8	16	4
IPM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	2	0.5	1	≤ 0.25
CFX	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
VCM	1	2	1	2	2	2	1	2	1
TEIC	≤ 0.5	1	≤ 0.5	1	1	1	1	1	1
DAP	1	> 4	0.5	1	1	2	1	2	2
GM	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
ABK	≤ 1	2	≤ 1	4	≤ 1	2	2	2	≤ 1
EM	1	0.5	> 4	> 4	4	> 4	> 4	> 4	> 4
MINO	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
LVFX	0.5	0.5	0.5	0.5	1	2	1	2	≤ 0.25
CLDM	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25
ST	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	> 2	> 2	1	≤ 0.5
RFP	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
LZD	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	2	2	2
FOM	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	32	≤ 16

Figure 1. Differences in MIC between DOT-MGA and DP42 for each condition (by strain).

Strains ATCC 29213 (a), ATCC 43300 (b), and KAM784 (c). MICs measured using DOT-MGA under each condition (inoculum size x 1/x 10, incubation time 6/8 hours) are compared with the DP42 results. Cells highlighted in dark blue indicate MICs ≥ 2 two-fold dilutions lower than DP42, light blue indicates 1 two-fold dilution lower, light pink indicates 1 two-fold dilution higher, and dark pink indicates ≥ 2 two-fold dilutions higher than the DP42 results. MIC: minimum inhibitory concentration, DOT-MGA: direct-on-target microdroplet growth assay, PCG: penicillin G, MPIPC: oxacillin, ABPC: ampicillin, S/A: sulbactam/ampicillin, CEZ: cefazolin, CFPM: cefepime, IPM: imipenem, CFX: cefoxitin, VCM: vancomycin, TEIC: teicoplanin, DAP: daptomycin, GM: gentamicin, ABK: arbekacin, EM: erythromycin, MINO: minocycline, LVFX: levofloxacin, CLDM: clindamycin, ST: sulfamethoxazole/trimethoprim, RFP: rifampicin, LZD: linezolid, FOM: fosfomycin.

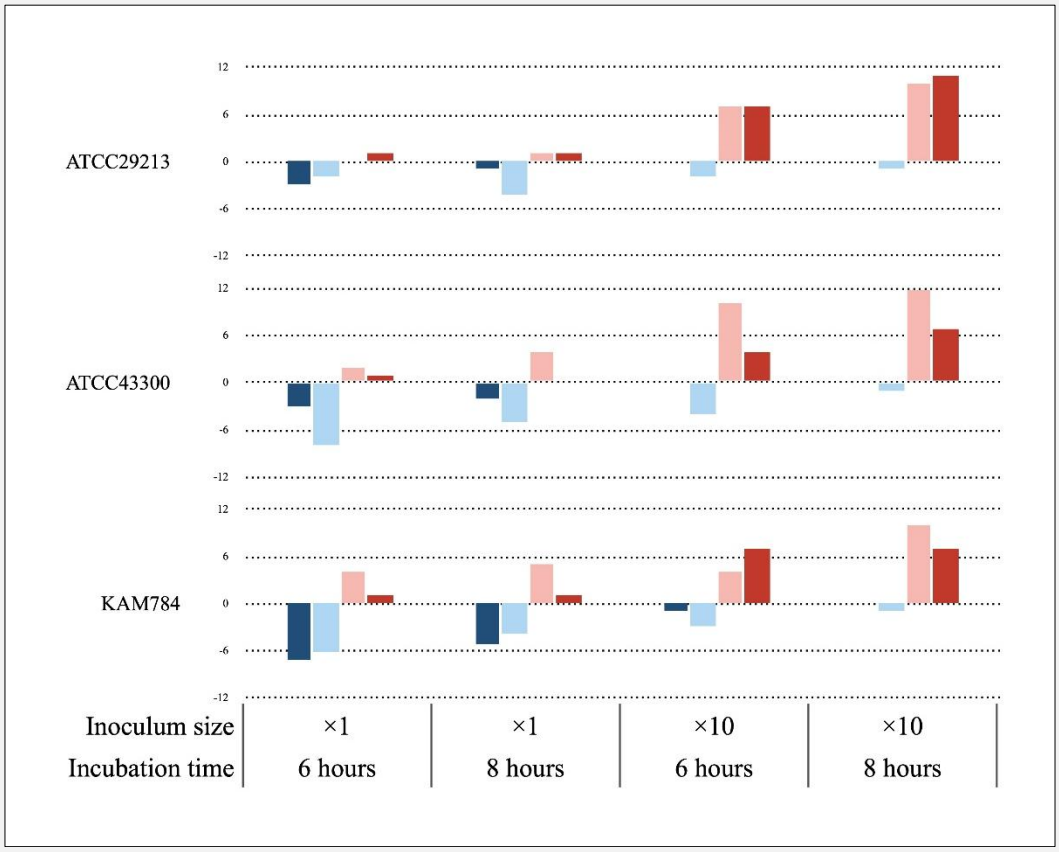


Figure 2. Agreement between DOT-MGA and DP42 across different conditions (by strain).

The vertical axis represents the cumulative number of cases (events). MICs under each condition (inoculum size x 1/x 10, incubation time 6/8 hours) are compared with DP42 data. Cells in dark blue indicate ≥ 2-fold MIC decrease, light blue indicates 1-fold MIC decrease, light pink indicates 1-fold MIC increase, and dark pink indicates ≥ 2-fold MIC increase. Data are presented for each strain: ATCC 29213, ATCC 43300, and KAM784. MIC: minimum inhibitory concentration, DOT-MGA: direct-on-target microdroplet growth assay, PCG: penicillin G, MPIPC: oxacillin, ABPC: ampicillin, S/A, sulbactam/ampicillin, CEZ: cefazolin, CFPM: cefepime, IPM: imipenem, CFX: cefoxitin, VCM: vancomycin, TEIC: teicoplanin, DAP: daptomycin, GM: gentamicin, ABK: arbekacin, EM: erythromycin, MINO: minocycline, LVFX: levofloxacin, CLDM: clindamycin, ST: sulfamethoxazole/trimethoprim, RFP: rifampicin, LZD: linezolid, FOM: fosfomycin.

In the present study, we also analyzed the agreement between the DOT-MGA and DP 42 methods using different antibiotics. While the standard inoculum generally yielded good concordance, some antibiotics exhibited MIC shifts regardless of the inoculum. For daptomycin, we observed elevated MICs under both inoculum conditions, possibly due to its membrane-disruptive mechanism, which may behave differently in short-term cultures. Linezolid exhibited consistently lower MICs for the DOT-MGA than for the DP42 method under all conditions. We considered two possible explanations. First, MALDI-TOF MS primarily detects ribosomal proteins [20], which may suppress protein expression earlier than expected, leading to earlier detection of growth inhibition. Second, linezolid exhibits variable MICs depending on the AST method [21], and its effect may be overestimated under short incubation times (6 - 8 hours), resulting in decreased MICs. Therefore, when DOT-MGA is applied to daptomycin or linezolid, the incubation time or scoring thresholds may require adjustment. Notably, our findings provide important insights into the relationship between the DP42 MIC range and actual MICs of the tested strains. At MICs values below the DP42 detection range, standard inoculum conditions exhibited agreement of > 90%, whereas high-inoculum conditions showed significantly lower concordance. This suggests that susceptible strains are more prone to inoculum effects, leading to false resistance. Conversely, when the MICs exceeded the DP42 range, high-inoculum conditions demonstrate enhanced

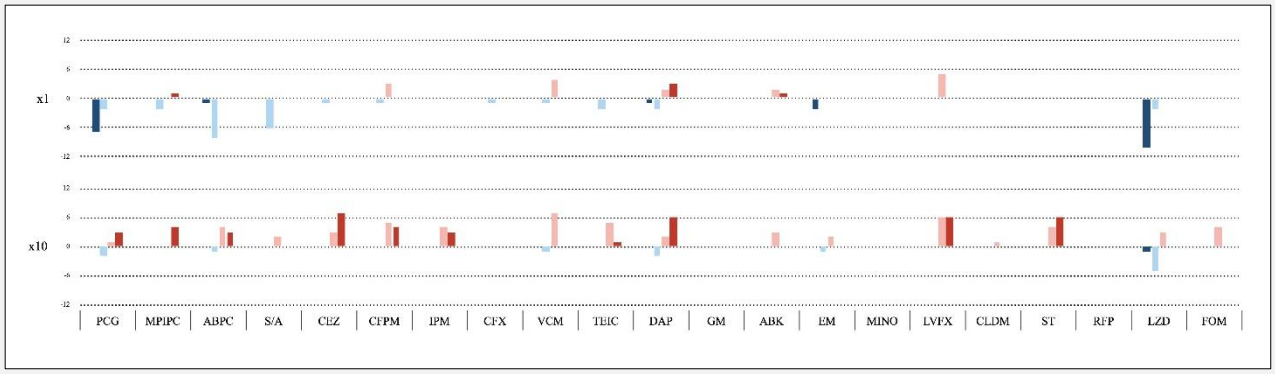


Figure 3. Impact of inoculum size on MIC consistency across antibiotics.

The vertical axis indicates the cumulative number of cases. MICs obtained using DOT-MGA under two inoculum conditions (standard x 1 and high inoculum x 10) are compared with the DP42 reference values. Cells in deep blue indicate MIC decreased by ≥ 2 dilutions, light blue indicates MIC decreased by 1 dilution, light pink indicates MIC increased by 1 dilution, and deep pink indicates MIC increased by ≥ 2 dilutions. MIC, minimum inhibitory concentration; DOT-MGA: direct-on-target microdroplet growth assay, PCG: penicillin G, MPIPC: oxacillin, ABPC: ampicillin, S/A: sulbactam/ampicillin, CEZ: cefazolin, CFPM: cefepime, IPM: imipenem, CFX: cefoxitin, VCM: vancomycin, TEIC: teicoplanin, DAP: daptomycin, GM: gentamicin, ABK: arbekacin, EM: erythromycin, MINO: minocycline, LVFX: levofloxacin, CLDM: clindamycin, ST: sulfamethoxazole/trimethoprim, RFP: rifampicin, LZD: linezolid, FOM: fosfomycin.

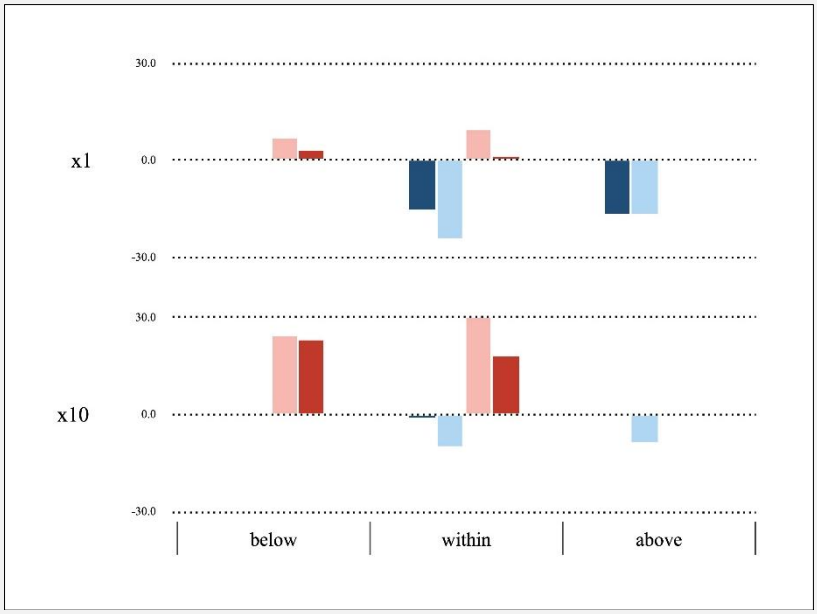


Figure 4. Concordance rate categorized by MIC range (under, within, above) and bacterial inoculum size (x 1, x 10).

The vertical axis represents the percentage relative to the total number of measurements. Cells are categorized according to the differences in MIC compared to the reference DP42 method: dark blue indicates a decrease of ≥ 2 two-fold dilutions, light blue indicates a decrease of 1 two-fold dilution, light pink indicates an increase of 1 two-fold dilution, and dark pink indicates an increase of ≥ 2 two-fold dilutions. MIC: minimum inhibitory concentration, DOT-MGA: direct-on-target microdroplet growth assay, PCG: penicillin G, MPIPC: oxacillin, ABPC: ampicillin, S/A: sulbactam/ampicillin, CE: cefazolin, CFPM: cefepime, IPM: imipenem, CFX: cefoxitin, VCM: vancomycin, TEI: teicoplanin, DAP: daptomycin, GM: gentamicin, ABK: arbekacin, EM: erythromycin, MINO: minocycline, LVFX: levofloxacin, CLDM: clindamycin, ST: sulfa-methoxazole/trimethoprim, RFP: rifampicin, LZD: linezolid, FOM: fosfomycin.

agreement, possibly owing to the enhanced growth of slow-growing resistant strains.

This study has some limitations. First, we tested a limited number of strains, which does not fully represent the diversity of the clinical isolates. Although the results were reproducible between the ATCC and a few clinical strains, the method must be validated in a larger and more diverse collection to confirm its generalizability. Second, the DP42 dry plate had a relatively narrow MIC range, which made interpretation difficult for highly susceptible or resistant strains. Broad-range reference methods, such as broth microdilution, are required for further validation. Third, we applied uniform conditions (inoculum and incubation time) for all antibiotics; however, optimal conditions may vary between drugs. For β-lactams, daptomycin, and sulfamethoxazole/trimethoprim, adjustments may be needed to avoid MIC over- or underestimation. Finally, we used the Biotyper score (≥ 1.7) as a threshold for positive growth; however, its standardization remains insufficient, as variations in spectral intensity due to species, drug type, or incubation time may influence scoring behavior. Recent advances in AI-based spectral analysis and automated growth classification may help improve the scoring systems for DOT-MGA.

In summary, we demonstrated that DOT-MGA is a promising platform for rapid AST. Further refinements and multicenter validations are needed to validate its utility in clinical settings. While most prior studies have focused on Gram-negative organisms, our study highlights the applicability of DOT-MGA to Gram-positive pathogens such as staphylococci. Future applications may be extended to vancomycin-resistant enterococci or penicillin-resistant *Streptococcus pneumoniae* to improve early decision-making in infectious disease management. In conclusion, DOT-MGA is a rapid, user-friendly, and flexible method that can be optimized in terms of inoculum size, incubation time, and drug characteristics. With further clinical validation and standardization, DOT-MGA holds great potential for adoption as a standard AST tool.

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The authors used OpenAI ChatGPT solely for language editing, and the authors take full responsibility for the final manuscript.

Declaration of Interest:

The authors declare that there are no conflicts of interest to disclose in relation to this work.

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