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Short Communication

Multicentric evaluation of the MTS™ Synergy Application System for reliable antibiotic synergy testing in clinical laboratories

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ABSTRACT

Objectives: The rise of antimicrobial resistance poses a major challenge for both clinicians and clinical microbiologists. There is an increasing need for user-friendly and reliable methods to assess the activity of antibiotics against multidrug-resistant (MDR) strains. Although synergy testing provides valuable insights, conventional methods such as checkerboard assays and time-kill studies are labour-intensive, technically demanding, and difficult to standardize. This study evaluated the MTS™ SAS (MIC Test Strip – Synergy Application System, Liofilchem, Italy), a commercial gradient diffusion assay developed for antibiotic synergy testing.
Methods: The performance of MTS™ SAS was evaluated in comparison with the checkerboard microdilution method, used as the reference standard. Nine antibiotic combinations were tested against ten different bacterial strains across 11 Italian hospitals. Inter-laboratory reproducibility and agreement with the reference method were analysed.
Results: The concordance between MIC test strips and the broth microdilution (BMD) method was 98.4%, with 1.6% showing discordant results – all within a 3-dilution range. Among 996 synergy determinations, MTS™ SAS demonstrated high reproducibility across all centers (96.7%), while only 3.3% of tests showed discordant synergy classifications (e.g., synergy vs. indifference). Comparison with the checkerboard method demonstrated an overall concordance of 96.2%, despite the absence of specific operator training at each site.
Conclusion: These findings support MTS™ SAS as a practical and reliable alternative to conventional synergy testing methods, particularly suitable for routine clinical settings and laboratories lacking advanced microbiological expertise.
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1. Introduction

The emergence of antimicrobial resistance (AMR) represents a critical threat to global public health and poses a growing chal-

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lenge for clinicians due to the diminishing availability of effective treatment options [1]. Among resistant pathogens, multidrug-resistant (MDR) Gram-negative bacteria are particularly concerning, especially in critically ill patients with multiple comorbidities [2]. The World Health Organization (WHO) has identified *Acinetobacter baumannii*, extended-spectrum β-lactamase producers Enterobacterales and carbapenem-resistant Enterobacterales (CRE) as part of the "critical priority" group of MDR organisms for which new therapeutic strategies are urgently needed [3].

Although recent years have seen the development of novel antimicrobial agents to counteract MDR pathogens [4], resistance to many of these new antibiotic combinations has already been reported, further limiting therapeutic options [5–7]. In this challenging context, antimicrobial combination therapy can be considered an alternative strategy to enhance treatment efficacy and delay the emergence of further resistance, particularly for infections caused by CRE [8,9].

Several in vitro methods are available to assess the synergistic effects of antibiotic combinations. Among these, the checkerboard assay is widely considered the reference method because of its conceptual simplicity, low equipment requirements, and standardized interpretation [10,11]. However, it is also labour-intensive, time-consuming, and resource-demanding, especially when multiple antibiotic combinations are tested simultaneously. Similarly, time-kill assays provide de tailed information on bactericidal activity over time but are technically demanding and often difficult to interpret consistently [12–15].

While gradient diffusion methods have been successfully used for determining minimum inhibitory concentrations (MICs) for over two decades, their application to synergy testing has been limited. This limitation is largely due to the diffusion characteristics of the antibiotics within the matrix. To address this, a commercial gradient diffusion-based system (MTS™ SAS - MIC Test Strip – Synergy Application System, Liofilchem, Italy) has been developed. This platform includes patented tools designed to simplify test performance and is further described at https://www.liofilchem.com/solutions/clinical/arm/mts-synergy-application-system.

The primary objective of this multicentre study was to assess the reliability, reproducibility, and inter-laboratory concordance of the MTS™ SAS in evaluating antibiotic synergy against MDR Gram-negative bacteria. The study was conducted across 11 Italian tertiary care hospitals, using a standardized protocol. No specific training was provided to participating laboratories, allowing us to assess the system's feasibility in real-world clinical microbiology settings.

2. Materials and methods

2.1. Bacterial strains

Bacterial isolates were selected based on the WHO priority list of drug-resistant pathogens posing the greatest threat to human health [3]. Species identification was carried out using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with the MALDI Biotyper system (Bruker Daltonik, Germany). Carbapenemase production was assessed through phenotypic methods and confirmed using the Xpert® Carba-R assay (Cepheid, USA) or, in the case of *Acinetobacter baumannii*, by an in-house PCR as previously described [16].

Ten non-duplicate strains were provided by the reference laboratory at IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, and distributed to 10 participating hospital microbiology laboratories across Italy (total of 11 centres). The isolates included: three *Pseudomonas aeruginosa* (two multidrug-resistant – MDR, harbouring both bla_{VIM} – a and one wild-type – WT); three *Acinetobacter baumannii* (two MDR, harbouring both $bla_{OXA\text{-}51}$ and $bla_{OXA\text{-}23}$ and one WT); three *Klebsiella pneumoniae* (one isolate harbouring bla_{VIM}, one bla_{KPC}, and the other harboring $bla_{CTX\text{-}M\text{-}15}$) and one MDR *Escherichia coli* (harbouring bla_{KPC}).

A full list of strains is provided in Table 1. These anonymized strains were collected from hospitalized patients admitted to the IRCCS Arcispedale Santa Maria Nuova of Reggio Emilia and have been previously characterized [16,17].

2.2. MICs determination

Preliminary antimicrobial susceptibility profiles were assessed using automated systems (Phoenix™ 100, Becton Dickinson, USA). MIC values were interpreted according to EUCAST clinical breakpoints (https://www.eucast.org/clinical_breakpoints).

Final MIC confirmation was performed by broth microdilution (BMD) at the reference laboratory following standardized protocols [18]. Briefly, serial two-fold dilutions of each antibiotic were prepared in Mueller-Hinton broth across a concentration range of 0.06 to 128 µg/mL. Bacterial suspensions were prepared from overnight cultures and adjusted to a 0.5 McFarland standard, then diluted in Mueller Hinton Broth to a final concentration of 5×10^5 CFU/mL. Microtiter plates were inoculated and incubated at 35 ± 2 °C for 18-24 hours and MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth. Each sample was tested in triplicate.

For the multicenter evaluation, each of the 11 participating laboratories tested MICs of ciprofloxacin, meropenem, colistin, ceftazidime, amikacin, and rifampin using commercial gradient diffusion strips (MIC Test Strip, Liofilchem, Italy). Tigecycline and rifampin were tested only against *A. baumannii* strains.

2.3. Checkerboard synergy testing

Antibiotic stock solutions were prepared by dissolving powders in sterile water to the appropriate concentrations, according to [12,18], and stored at -80 °C. For checkerboard assays, a 96-well plate was filled with 50 μL of Mueller-Hinton broth per well.

Antibiotic A was serially diluted along the vertical axis; antibiotic B along the horizontal axis.

Inoculum was prepared to a 0.5 McFarland standard and diluted to 5×10^5 CFU/mL, and each well was filled with 100 μL of the bacterial suspension. Plates were incubated at 35-37 °C for 24 hours under aerobic conditions. The MIC of each antibiotic in combination was recorded, and synergy was evaluated by calculating the Fractional Inhibitory Concentration (FIC) Index (FICI).

Briefly, the FICI was calculated as follows: FICI = FIC of agent A (FIC A) + FIC of agent B (FIC B), where FIC A is the MIC of the combination/MIC of drug A alone, and FIC B is the MIC of the combination/MIC of drug B alone. FICI results were interpreted as: synergy, FICI $\leq$ 0.5; indifferent, 0.5 $>$ FICI $\leq$ 4; antagonism, FICI $\geq$ 4 [11,14].

2.4. MTS™ SAS synergy testing

Synergy testing was performed using the MTS™ SAS and the antibiotic combinations examined are shown in Table 2.

Each bacterial isolate received from the coordinator center was revitalized through subcultures performed on Columbia blood agar and MacConkey agar and checked for purity. Bacterial suspensions were prepared by resuspending each strain, subcultured overnight on Mueller-Hinton agar, in a saline solution (NaCl 0.9%) to a final turbidity equal to 0.5 McFarland (McF) standard. Then, the suspensions were plated uniformly on MH agar plate using a swab soaked in saline bacterial suspension. For each drug combination to be tested, a single strip of each antibiotic was positioned on

Table 1
List of the isolates and antibiotic tested. Right part: MICs values expected and considered acceptable (based on broth microdilution assays (see text).

Strain	Species	B-lactamase	CIP	MRP	CS	TGC	CAZ	AK	RD
AC73	*Acinetobacter baumannii*	bla_{OXA-51} + bla_{OXA-23}	>32	>32	0.5-2	1-4	>256	32-128	4-16
AC74	*Acinetobacter baumannii*	bla_{OXA-51} + bla_{OXA-23}	>32	>32	0.5-2	1-4	16-64	0.5-2	2-8
AC71	*Acinetobacter baumannii*	WT	0.125-0-5	0.25-1	0.5-2	0.125-0.5	2-8	0.5-2	4-16
CIP1	*Pseudomonas aeruginosa*	bla_{VIM-1}	>32	>32	1-4	NT	>256	8-32	NT
CIP40	*Pseudomonas aeruginosa*	WT	0.25-1	0.25-1	1-4	NT	1-4	4-16	NT
CIP10	*Pseudomonas aeruginosa*	bla_{VIM-1}	>32	>32	1-4	NT	1-4	4-16	NT
CRE28	*Klebsiella pneumoniae*	bla_{KPC-2}	>32	>32	0.5-2	NT	>256	32-128	NT
CRE33	*Klebsiella pneumoniae*	bla_{KPC-3}	>32	0.5-2	0.25-1	NT	4-16	1-4	NT
CRE7	*Klebsiella pneumoniae*	$bla_{CTX-M-15}$	>32	1-4	0.5-2	NT	>256	>256	NT
-1921	*Escherichia coli*	bla_{KPC-3}	8-32	0.03-0.125	0.25-1	NT	8-32	1-4	NT

Abbreviations: CIP = ciprofloxacin, MRP = meropenem, CS = colistin, TGC = tigecycline, CAZ = ceftazidime, AK = amikacin, RD = rifampin, WT = wild type.

Table 2
List of errors type on MICs determination by MIC test strips in comparison to the reference microdilution method for different antimicrobials included in this study.

Antibiotics	Errors						MIC value (>$\log_2$)	
	mE (n)	%	ME (n)	%	VME (n)	%	(n)	%
Ciprofloxacin	1	0.9%	0	0%	0	0%	3	2.5%
Meropenem	14	11.6%	0	0%	0	0%	10	8.3%
Colistin	0	0%	0	0%	0	0%	10	8.3%
Tigecycline	NA	NA	NA	NA	0	0%	5	13.8%
Ceftazidime	2[b]	2.4%[b]	0[b]	0%[b]	0	0%	3	2.5%
Amikacin	9	7.5%	6	5%	1	0.9%	2	1.7%
Rifampicin	NA	NA	NA	NA	0	0%	0	0%
Total	26	4.6%	6	1%	1	0.2%	33	4.9%

Abbreviations: mE = minor errors, Susceptible or Resistant by BMD and intermediate by gradient tests; MR = major errors; Susceptible by BMD and resistant by Strips; VME, very major errors; Resistant by BMD and susceptible by Strips; NA; not applicable

the plates inoculated with bacteria suspension incubated to 16-20 hours to 37°C, thus determining the MICs of the single drugs.

The MTS™ SAS test was performed according to the manufacturer's instructions.

After MIC determination of single antibiotics, two MTS™ strips, each containing one of the antibiotics to be tested, were placed perpendicular to each other in the MTS™ Synergy Applicator System, intersecting at the MIC for each antimicrobial when tested individually. The two strips were then picked up using the MTS synergy delivery tool moving them on the surface of a Mueller-Hinton agar plate inoculated with a suspension of the strain. The strips were then pushed onto the agar surface by using tweezers to facilitate their removal of the MTS Synergy Delivery Tool.

Plates were incubated at 37 °C for 16–20 hours. Synergy was assessed by evaluating the shape of the inhibition ellipse at the intersection point and calculating the FICI, as described for the checkerboard method. Antibiotic combinations tested are listed in Table 2.

2.5. Data analysis

MIC values obtained using the gradient diffusion test at the 11 centres were compared with BMD results. Agreement was considered acceptable if MIC values were within ±1 dilution.

The results of synergy tests have been stratified in accordance with the FIC index, and according to Odds the antimicrobial combinations have been considered synergistic if FIC ≤0.5; antagonistic if >4.0, and indifferent if falling between these two categories [14]. Additionally, two centres (Reggio Emilia and Bologna) performed parallel checkerboard testing to assess inter-laboratory reproducibility and concordance with MTS™ SAS results. Concordant results were defined as those classified into the same FICI category by both methods.

Concordance and reproducibility were assessed by calculating the percentage of agreement among laboratories and with the reference method. In addition, Cohen's kappa coefficient (κ) was calculated to evaluate the strength of agreement beyond chance, and McNemar's test was applied to assess the statistical significance of paired categorical differences between methods. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Bacterial characterization

Ten clinical strains with different susceptibility profiles to antimicrobial molecules were selected. The genotypic and phenotypic traits of MDR isolates are shown in Table 1.

3.2. MICs determination

All 11 participating centres tested five antibiotics against each of the three *Pseudomonas aeruginosa*, three *Klebsiella pneumoniae*, and one *Escherichia coli* strains, resulting in 35 MIC determinations per centre. Seven antibiotics were tested against each of the three *Acinetobacter baumannii* isolates, yielding 21 additional determinations per centre. This resulted in a total of 56 MICs per laboratory and 616 MIC data points across all sites.

Across the dataset, a systematic overestimation of colistin MICs by the gradient diffusion method was observed when compared to the BMD reference method. However, this discrepancy did not result in any change in categorical interpretation (i.e., susceptible vs. resistant). A summary of MIC discrepancies between MTS™ and BMD for each laboratory is provided in Table 2.

Despite minor discrepancies, overall concordance with BMD (defined as MIC results within ±1 dilution) was 98.4%. Only 10 determinations (1.6%) fell outside the three-dilution agreement range. Notably, two of these discrepancies involved meropenem MICs for a *K. pneumoniae* strain harbouring bla_{KPC}, which exhibited heteroresistance.

3.3. Synergy testing

For synergy testing, all 11 centres assessed 9 antibiotic combinations against the three *P. aeruginosa*, three *K. pneumoniae* and

Table 3
Antibiotics combinations tested, MTS^{TM} SAS interlaboratory agreement among the different centres and concordance with checkerboard method. Agreement was defined as the same categorization proposed by the centre on the basis of the FIC index.

Antibiotic combinations	Total tests	MTS-SAS agreement	
		among sites (nr - %)	to checkerboard (%)
Colistin + meropenem	105	104 (99.0)	99.2
Meropenem + ciprofloxacin	105	100 (95.2)	95
Amikacin + ceftazidime	108	97 (89.8)	92
Colistin + ceftazidime	109	108 (99.1)	99.2
Colistin + ciprofloxacin	109	109 (100)	100
Amikacin + meropenem	104	96 (92.3)	75.9
Amikacin + ciprofloxacin	108	108 (100)	100
Ceftazidime + ciprofloxacin	108	102 (94.4)	90
Colistin + amikacin	109	108 (99.1)	99.2
Tigecycline + rifampin*	31	31 (100)	100
TOTAL	996	963 (96.7)	95.3

* Combination used only for testing *A. baumannii* strains.

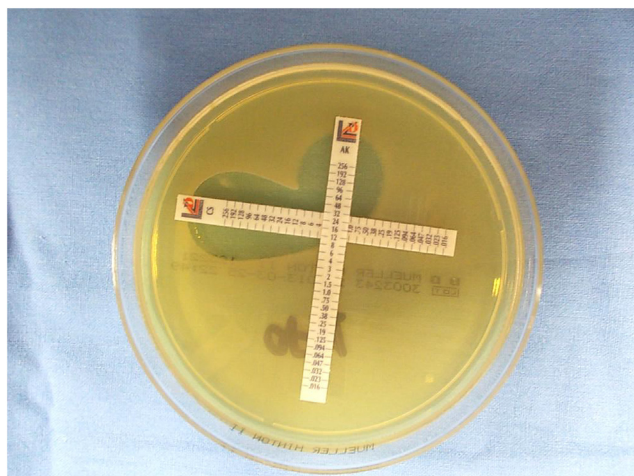

Fig. 1. Synergy testing with MTS-SAS^{TM}: example of indifference.

Fig. 2. Synergy testing with MTS-SAS^{TM}: example of synergic effect. The arrow highlights the inhibition area and the reduced MIC of the meropenem in combination with amikacin for a strain of *K. pneumoniae*.

one *E. coli* (i.e., 693 determinations) and 10 combinations against the three *A. baumannii* isolates (i.e., 330 determinations).

In total, 1,023 synergy determinations were conducted using the MTS^{TM} SAS system. Representative results showing an indifferent effect and a synergistic effect are presented in Figures 1 and 2, respectively.

Twenty-seven synergy tests involving strains with MIC values not in agreement with the BMD reference method (as noted above) were excluded from the final analysis. The remaining dataset included 996 valid drug combination results.

Inter-laboratory agreement of MTS^{TM} SAS synergy results is summarized in Table 3. Discordant results were observed in 33 cases (3.3%), all involving differences in synergy classification (synergy vs. indifference); no cases of antagonism were recorded. The most inconsistent combinations were amikacin + ceftazidime (89.8%), and amikacin + meropenem (92.3%). Importantly, 19 of the 33 discordant cases involved a single isolate, the *P. aeruginosa* wild-type isolate.

When compared to the checkerboard method (performed by two reference laboratories), MTS^{TM} SAS demonstrated an overall concordance of 96.2%. Cohen's kappa coefficient ($\kappa = 0.91$, $p < 0.001$) indicated an excellent level of agreement beyond chance, and McNemar's test did not show a statistically significant difference between the two methods ($p = 0.42$). The greatest disagreement between the two methods was observed for amikacin + ceftazidime and ceftazidime + ciprofloxacin, where synergy was more frequently detected with the MTS^{TM} SAS method, while the checkerboard assay indicated indifference (Figs 1 and 2).

4. Discussion

In this study, we presented the *in vitro* antimicrobial activity of various antimicrobial combinations against a panel of MDR microorganisms using a commercial synergy testing assay. Historically, gradient diffusion synergy testing involved a cumbersome procedure, where two antibiotic strips were placed on an agar plate inoculated with a lawn of the test organism. After allowing the first strip (containing the first antibiotic) to act for 60 minutes, it was removed, and the second strip (containing the second antibiotic) was placed in the same position. This approach, however, was largely abandoned due to the technical challenges it posed, leading to poor reproducibility [11].

This study demonstrates that the MTS^{TM} SAS gradient diffusion method is a reliable, reproducible, and user-friendly system for antimicrobial synergy testing, even in settings without specialized training. The high concordance (98.4%) of MIC determinations with the broth microdilution (BMD) reference method highlights the accuracy of the MTS^{TM} SAS system in antimicrobial susceptibility testing. Furthermore, synergy testing using MTS^{TM} SAS showed an overall inter-laboratory agreement of 96.2% when compared with the checkerboard microdilution method, validating its ability to provide consistent results across different clinical laboratories.

To our knowledge, this is the first multicentric evaluation of the *in vitro* antibacterial activity of different antimicrobial molecules

using a commercial synergy testing solution. Specifically, we evaluated synergy testing against MDR microorganisms belonging to bacterial species identified by WHO as critical priorities for new antibiotic development [3].

However, this study has some limitations. The number of strains analysed is relatively small, and the range of antimicrobial agents tested could be expanded. This limited strain panel, although designed to ensure inter-laboratory feasibility, may restrict the generalizability of our findings, and potential strain selection bias or strain-specific variability cannot be completely excluded. Moreover, although the isolates included represent clinically relevant MDR pathogens, they did not encompass the most challenging resistance phenotypes, such as extensively or pan-resistant strains or those harbouring multiple resistance mechanisms. This may limit extrapolation of our results to highly resistant clinical scenarios, where synergy testing could be particularly beneficial. Future studies including such phenotypes will be valuable to further assess the robustness and clinical performance of the MTS™ SAS system. Further evaluations – whether from experimental studies or real-world applications – should also encompass a broader range of bacterial species and newly developed antimicrobial agents.

Furthermore, we chose to include colistin, a drug that EUCAST advises against testing with gradient methods, to assess the robustness of the experiments. Minor discrepancies were observed, particularly an overestimation of colistin MICs, but these did not affect the clinical categorization of the strains, further supporting the clinical applicability of the MTS™ SAS system.

Although the operators did not receive specific training before performing the tests, this condition was intentionally maintained to reflect real-world laboratory practices. While this approach strengthens the assessment of the method's routine applicability, it may also introduce a degree of operator-related variability. Nonetheless, the high inter-laboratory concordance observed suggests that such variability had a limited impact on overall reproducibility. Moreover, in practical settings, laboratories implementing the MTS™ SAS would provide specific operator training, which would further reduce potential variability and procedural errors.

The synergy testing results demonstrated that MTS™ SAS can effectively identify synergistic interactions between antibiotic combinations, offering an alternative to traditional, more resource-intensive methods like checkerboard and time-kill assays. Importantly, this method's simplicity and ease of use make it an ideal tool for widespread adoption, particularly in resource-limited settings or laboratories lacking specific expertise in complex antimicrobial testing procedures. Its straightforward workflow enables implementation within routine laboratory procedures, potentially reducing turnaround time for synergy assessment. By providing timely and reproducible results, MTS™ SAS may support earlier optimization of antimicrobial therapy and more informed clinical decision-making, thereby enhancing patient management in cases of multidrug-resistant infections. Given the ongoing global threat posed by multidrug-resistant organisms, methods like MTS™ SAS can play a key role in optimizing combination therapy regimens, potentially reducing the emergence of resistance and improving patient outcomes. Further studies and validation in diverse clinical settings will help refine the method's application and assess its full potential in routine microbiological diagnostics.

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Ethical approval: Not required.

Data availability: Strains are available upon valuable request

Declaration of competing interest: None of the investigators of the different hospitals which took part in the study have any kind of financial involvement with Liofilchem S.r.l.

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