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#### ARTICLE TITLE

When one drug is not enough: Context, methodology, and future prospects in antibacterial synergy testing

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## KEYWORDS

Antimicrobial synergy, synergy testing, antimicrobial susceptibility testing, antimicrobial resistance, checkerboard array, time-kill assay, hollow fiber infection model

## KEY POINTS

- Antibacterial combinations are used in clinical practice to accomplish a variety of therapeutic goals, including prevention of resistance and enhanced antimicrobial activity.
- The most common types of synergy testing are the checkerboard array assay, the time-kill study, diffusion assays, and pharmacokinetic/pharmacodynamic models such as the hollow fiber infection model.
- Antibacterial synergy testing is not routinely performed in the clinical microbiology laboratory because of test complexity and uncertainty about the predictive value of synergy testing results for patient outcomes.
- Optimized synergy testing techniques and better data on the relationship between *in vitro* synergy results and clinical outcomes are needed to guide rational use of antimicrobial combinations in the multidrug resistance era.

## SYNOPSIS

Antibacterial combinations have long been used to accomplish a variety of therapeutic goals, including prevention of resistance and enhanced antimicrobial activity. *In vitro* synergy testing methods, including the checkerboard array, the time-kill study, diffusion assays, and pharmacokinetic/pharmacodynamic models, are used commonly in the research setting, but are not routinely performed in the clinical microbiology laboratory because of test complexity and uncertainty about their predictive value for patient outcomes. Optimized synergy testing techniques and better data on the relationship between *in vitro* results and clinical outcomes are needed to guide rational use of antimicrobial combinations in the multidrug resistance era.

## INTRODUCTION

Combination antibacterial therapy dates back to the early antibiotic era<sup>1</sup> and remains a common practice today. Some combinations have been widely used for decades, their clinical utility well supported by clinical outcomes data, while others have only recently been described in *in vitro* studies. A number of different techniques are used in the laboratory to test for synergistic activity in drug combinations. To understand the utility and limitations of these testing methods, we will evaluate them in the context of the mechanisms they are designed to test and the various clinical rationales for antibacterial combination therapy.

## MECHANISMS OF COMBINATION ANTIBIOTIC ACTIVITY

In order to make sense of synergy testing methods, it is essential to understand the mechanisms by which antibiotics can work when used in combination. There are two main conceptual reasons for using drugs in combination. The first is to prevent the emergence of resistance to any individual drug during treatment. Regimens used to prevent resistance will be discussed briefly below, in the context of *Mycobacterium tuberculosis*, but are not otherwise a primary focus in this review, as they do not rely on synergy in the sense of enhanced combinatorial activity, and their efficacy is not evaluated using synergy testing. The other reason to use two or more antibiotics together is that some drugs, in combination, exhibit activity that is greater than would be expected through simple additive activity: in other words, they are synergistic.

### Prevention of Resistance

Discovered in 1943, streptomycin was the first drug in history with activity against *M. tuberculosis*,<sup>1,2</sup> an ancient and deadly disease that has plagued humankind for thousands of years.<sup>3</sup> But it soon became apparent that *M. tuberculosis* isolates from patients treated with streptomycin alone developed resistance to the drug during therapy;<sup>4</sup> only when multiple anti-tuberculosis drugs were used in combination could resistance to any one of the agents reliably be prevented during the prolonged treatment courses required to cure tuberculosis.<sup>1</sup> Today, the standard initial regimen for drug-susceptible *M. tuberculosis* isolates is a combination of four drugs: isoniazid, rifampin, ethambutol, and pyrazinamide.<sup>5</sup> Such multidrug regimens are essential for effective treatment because *M. tuberculosis* develops resistance to each of these drugs relatively simply, through spontaneous chromosomal mutations.<sup>6</sup> As a result, during a standard course of tuberculosis therapy, which lasts at least six months,<sup>5</sup> the chance of an organism developing resistance to a drug used as monotherapy may be as high as 100%,<sup>7</sup> while the likelihood of an organism simultaneously developing resistance to four drugs is vanishingly small.

### Enhanced Activity

Most bacterial pathogens do not develop resistance to antibiotics in as simple a manner as *M. tuberculosis*,<sup>8</sup> and the use of combination regimens has not been shown to be an effective method for the prevention of resistance in organisms such as *Enterobacteriaceae*.<sup>9</sup> Instead, the primary rationale for the use of combination regimens in most bacterial

pathogens is to overcome an existing resistance mechanism or to improve the activity of one or both agents<sup>10</sup> (see Table 1).

Table 1 Mechanisms of Synergistic Antibacterial Activity			
Mechanism	Example(s)	Explanation	Clinical testing method
Inhibition of sequential steps in a biosynthetic pathway	Sulfamethoxazole + trimethoprim	Sulfamethoxazole and trimethoprim act synergistically <sup>11</sup> by inhibiting different steps in the production by bacteria of tetrahydrofolic acid, a key component in numerous bacterial biosynthetic processes. <sup>12</sup>	Drugs tested as a combination using standard AST
Inhibition of resistance mechanisms	Ampicillin-sulbactam, ceftazidime-avibactam	A $\beta$ -lactam antibiotic combined with a $\beta$ -lactamase inhibitor that protects the antibiotic from destruction by bacterial $\beta$ -lactamase enzymes. <sup>13</sup>	Drugs tested as a combination using standard AST
Increased entry into the bacterial cell	Ampicillin or vancomycin + gentamicin for <i>Enterococcus</i>  Colistin + drugs with limited ability to cross outer membrane (e.g. linezolid) <sup>14</sup>	Aminoglycosides (e.g. gentamicin) are clinically ineffective against <i>Enterococcus</i> species due to limited ability to enter the bacterial cell at <i>in vivo</i> concentrations. When given with an enterococcal cell wall-active drug (e.g. ampicillin, vancomycin), gentamicin uptake is increased and its concentration at the ribosomal target reaches levels necessary for activity. <sup>15</sup>  Colistin and related compounds permeabilize the outer membrane of Gram-negative bacteria. <i>In vitro</i> synergy between these agents and antibiotics that are not normally active against Gram-negative bacteria <sup>14,16,17</sup> is believed to result from increased entry into the cell. <sup>16</sup>	HLAR (High-level Aminoglycoside Resistance) testing  Not performed
Double $\beta$ -lactam therapy for <i>Enterococcus</i>	Ampicillin + ceftriaxone for enterococcal endocarditis <sup>18</sup>	Complete saturation of non-essential penicillin-binding proteins (PBPs) by cephalosporins, which are ineffective against enterococci as monotherapy, in combination with partial saturation of essential PBPs by ampicillin, is believed to be mechanism of synergy. <sup>19</sup>	Not performed
Enhancement of biofilm activity	Addition of rifampin to regimens for staphylococcal prosthetic material infections (including prosthetic valve endocarditis) <sup>20,21</sup>	Rifampin, an inhibitor of bacterial DNA-dependent RNA polymerase, is almost never used as monotherapy because most bacteria rapidly develop resistance during therapy. <sup>22</sup> However, it is particularly active against biofilms and is often used for this purpose in combination with other drugs.	Not performed ( <i>Staphylococcus</i> species tested for rifampin susceptibility by standard AST)
Unknown mechanisms	Amikacin + doripenem for carbapenemase-producing <i>K. pneumoniae</i> <sup>23</sup> Meropenem + levofloxacin for <i>P. aeruginosa</i> <sup>24</sup> Fosfomycin +	Using <i>in vitro</i> synergy methods and animal models, numerous antibiotic combinations have been tested against multidrug-resistant pathogens for which few standard treatment options exist, such as carbapenem-resistant <i>Enterobacteriaceae</i> (CRE), <sup>23,26,27</sup> <i>Pseudomonas aeruginosa</i> , <sup>24,28</sup> <i>Acinetobacter baumannii</i> , <sup>29</sup> and vancomycin-resistant enterococci. <sup>25,30</sup> Few studies have evaluated synergy mechanisms for these combinations.	Not performed

	daptomycin for vancomycin-resistant <i>Enterococcus</i> <i>faecium</i> <sup>25</sup>		
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## SYNERGY TESTING METHODS

Several different methods are commonly used to test for synergy. These methods do not always yield identical results,<sup>31–33</sup> but determining which is most reliable is challenging, as there is no established gold-standard synergy reference method. An ideal technique would consistently predict treatment outcomes, but to date there have been few comparisons of *in vitro* synergy testing data with clinical outcomes; furthermore, different methods may work best for different organisms or drugs.<sup>34</sup>

### Checkerboard array

The checkerboard array method is an adaptation of standard broth-based minimal inhibitory concentration (MIC) testing. As such, it can assess inhibition of bacterial growth, but does not provide information on bacterial killing.<sup>35</sup> The array is typically created in a 96-well microtiter plate, with each well containing a standardized bacterial inoculum and appropriate concentrations of antibiotics,<sup>35</sup> although adaptations using automated dispensing and smaller volumes have been described.<sup>27</sup> A typical checkerboard array layout is shown in Figure 1. Microplates are examined for evidence of growth after incubation under standard antimicrobial susceptibility testing (AST) conditions.<sup>36</sup>

The results of a checkerboard array synergy assay are evaluated by calculating the fractional inhibitory concentration (FIC) index. In a well in which growth is inhibited, the FIC of each drug is determined by dividing the concentration of the drug in that well by the MIC of that drug alone.<sup>35</sup> The FIC index is the sum of the FIC values of two drugs in a well. An FIC index of  $\leq 0.5$  is considered synergistic, while an FIC index of  $> 4.0$  is antagonistic and intermediate values are considered to show no interaction.<sup>37</sup> It should be noted that to meet the definition of synergy, each drug must be present at *less than half* its MIC. This definition reflects the expected variability of  $\pm 1$  two-fold dilution in single-agent MIC testing:<sup>38</sup> an FIC index of 1 could simply result from both drugs demonstrating inhibition at one-half their respective MICs by chance. While the FIC index is the most common measure of synergy in the checkerboard array, other models have been developed.<sup>39</sup> In addition, the checkerboard array can be used to test more than two drugs at once,<sup>40,41</sup> although this method quickly becomes impractical unless limited concentration ranges are used for one or more of the drugs being tested.

#### Diffusion-based methods

Like the checkerboard array, diffusion-based synergy methods provide information about bacterial inhibition but not about killing. Disk diffusion synergy testing is based upon the principle of disk diffusion AST, in which a paper disk impregnated with the antibiotic of interest is placed upon a lawn of bacteria on an agar plate. The drug diffuses through the agar and the diameter of the zone of bacterial clearance around the disk is measured after incubation and compared to established breakpoint tables to determine whether the

organism is susceptible to the drug.<sup>36,42</sup> When this method is adapted for synergy testing, disks containing two different drugs are placed on a plate; if there is enhanced clearance or bridging between the two zones, the combination is considered synergistic, whereas if there is decreased clearance between the two zones, it is considered antagonistic.<sup>43,44</sup> Although this method is relatively simple to perform, it is not described often in the literature, perhaps because of concerns about subjectivity and a lack of established data.<sup>10,43,45</sup>

Antibiotic gradient diffusion strips work by a similar principle, except that the gradient of drug in the strip allows for determination of an MIC value based on the point at which the ellipse of growth inhibition intersects with the strip.<sup>46</sup> Several methods for gradient strip synergy testing have been described, including placement of strips at a right angle intersecting at the point of their relative MICs<sup>33</sup> and placing a strip containing one drug onto the agar in a location on which a strip containing the other drug had diffused before being removed.<sup>47</sup> The concentration at which each drug inhibits growth in the combination configuration is assessed after incubation, and these values are used to calculate an FIC index.<sup>33,47</sup>

#### Time-kill assay

The time-kill synergy assay is more labor-intensive than the checkerboard array, and final results, which are dependent on growth of bacterial samples for quantification, are delayed by a day compared to the checkerboard array. However, time-kill assays provide information not only on synergy but also on the time course of bacterial growth and on



bactericidal activity. In a time-kill synergy study, bacteria are incubated in liquid culture tubes under the following conditions: each antibiotic alone at a given concentration, the two antibiotics in combination at the same concentrations, and an antibiotic-free growth control.<sup>48</sup> Aliquots are removed from each tube for colony enumeration at the beginning of the experiment, at interval time points, and at 24 hours. If the colony count at 24 hours from the combination tube is  $\geq 2 \log_{10}$  less than the count from the tube containing the most active drug alone, the combination is synergistic.<sup>49</sup> If the colony count at 24 hours from the combination tube is  $\geq 3 \log_{10}$  less than the starting inoculum, then the combination is also bactericidal.<sup>49</sup>

It should be noted that the nature of the assessment of synergy is different in time-kill and checkerboard array studies. The time-kill study compares the same concentrations of antibiotic together and separately and evaluates whether the combination is *more* effective at 24 hours than either individual drug. By contrast, because the checkerboard array has a binary outcome measure (inhibition of bacterial growth as detected by the absence of visually apparent turbidity), it can only assess whether a given concentration combination is effective or not. A checkerboard array study answers the question, “By how much can drug concentrations can be reduced while still inhibiting growth?”, whereas a time-kill study answers the question, “How much more effective is the combination than its constituent drugs?”

In vitro pharmacokinetic/pharmacodynamic (PK/PD) models

*In vitro* PK/PD models differ from other synergy assays in that antibiotic concentrations can be varied over time, mimicking tissue drug concentrations during antibiotic therapy and allowing for simulation and comparison of different dosing regimens.<sup>50</sup> One of the most widely adopted PK/PD systems is the hollow-fiber infection model (HFIM).<sup>51–53</sup> The HFIM includes a central compartment, representing the circulatory system, from which media containing varying concentrations of antibiotics is pumped continuously through semipermeable fibers in a capillary unit similar to a dialysis cartridge. The antibiotic diffuses through pores in the fibers into the peripheral compartment, which is inoculated with bacteria to represent a site of infection. Because bacteria are too small to pass through the pores, antibiotic concentrations can be changed without artificially changing the bacterial concentration. In addition, bacterial waste products can diffuse out through the pores, allowing for experimental durations of two weeks or more.<sup>54</sup> Such durations facilitate evaluation of the emergence of resistance during treatment.<sup>55</sup>

### Animal models

While *in vitro* PK/PD models can mimic *in vivo* drug concentrations, there are features of *in vivo* infection, such as immune response and tissue environment, that cannot be fully replicated in artificial systems. Animal infection models, usually in mice, allow for synergy testing in a living organism of different types of infection, including thigh infection,<sup>56</sup> sepsis,<sup>57</sup> and pneumonia.<sup>23</sup> The significant physiological differences that exist between model organisms and humans must be accounted for to the extent possible in these

models, for example by inhibiting murine renal function to mimic human drug metabolism<sup>58</sup> or inducing neutropenia to increase susceptibility to infection.<sup>59</sup>

## SYNERGY TESTING IN THE CLINICAL MICROBIOLOGY LABORATORY

It is notable, given this variety of well-established methods, that synergy testing is not routinely performed on organisms from patient samples in the clinical microbiology laboratory. There are two reasons for this. First, most synergy methods are too labor-intensive to be incorporated into the clinical laboratory workflow. Second, as will be discussed in more detail below, there is considerable debate about the direct clinical applicability of synergy testing results, and interpretive criteria for synergy testing have not been adopted by the Clinical and Laboratory Standards Institute (CLSI).

For a few well-established combinations with known mechanisms of synergy, however, standardized susceptibility testing methods with published interpretive criteria allow the clinical laboratory to assess the activity of the combination without performing a full synergy assay.<sup>36</sup> To test for acquired high-level aminoglycoside resistance (HLAR), which is the primary mechanism of resistance to synergy between an aminoglycoside and a cell wall-active agent in *Enterococcus* species,<sup>60</sup> enterococci are exposed to very high levels of gentamicin (500 µg/mL) or streptomycin (1000 µg/mL).<sup>36</sup> Isolates with HLAR are not inhibited by these concentrations, and such resistance predicts a lack of synergistic aminoglycoside activity against the isolate.<sup>61</sup> Most drugs that are used together for synergy, including β-lactam-β-lactamase inhibitor combinations and trimethoprim-sulfamethoxazole,

are manufactured and administered to patients as combination products at fixed ratios,<sup>11,13</sup> and the laboratory simply tests these combinations using standard AST methods.

#### CLINICAL RELEVANCE OF *IN VITRO* SYNERGY TESTING RESULTS

A few commonly used drug combinations, including  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations and trimethoprim-sulfamethoxazole, have been investigated in clinical studies, and their efficacy and advantages over single-agent therapy for appropriate infections have been well established.<sup>62,63</sup> However, while dozens of different drug combinations have been tested *in vitro* against various different species of bacteria,<sup>64,65</sup> there remains uncertainty about the clinical relevance of these results. Combinations often appear to be synergistic against some isolates and not others,<sup>66,67</sup> but it is unclear whether this is due to variable efficacy of the combination from one strain to another, in which case testing of individual patient isolates might be indicated, or to variability in methods or techniques, in which case method standardization and development of more robust synergy testing techniques may be required. At the heart of the uncertainty about clinical applicability of synergy testing lies the paucity of data incorporating both *in vitro* and clinical data from the same isolates. With a few exceptions in the form of case reports and small series,<sup>52,68–70</sup> most clinical studies are retrospective investigations that do not include *in vitro* synergy data;<sup>71,72</sup> as a result, it is impossible to know whether such results could have predicted either the overall efficacy of the combination or its utility for specific patients.

One randomized, double-blind, controlled trial has been performed to evaluate the effect on patient outcomes of combination antibiotic testing to guide drug selection.<sup>73</sup> This study compared outcomes in patients with cystic fibrosis (CF) treated with antibiotic regimens selected based on *in vitro* multiple combination bactericidal antibiotic testing (MCBT) to those treated with regimens selected based on standard AST. The authors found no difference in time to next pulmonary exacerbation between the two treatment groups. Possible explanations for the lack of benefit from combination testing in this study include limitations in the applicability of *in vitro* AST results for isolates from patients with CF and the fact that the susceptibility of many of the isolates to different combination regimens had changed from the time of combination testing to the time of treatment,<sup>74,75</sup> but it is also important to note that MCBT lacks one of the key characteristics of the synergy testing methods described earlier: it does not compare the activity of a combination to the activity of its constituent components.<sup>73</sup> In MCBT, clinically relevant concentrations of antibiotics are combined and incubated with the bacterial isolate, and if no visible turbidity is detected after a fixed period of incubation, a colony count is performed to assess for bactericidal activity. However, if one of the drugs in a combination is bactericidal on its own, then it is not clear that improved activity would be expected from use of a bactericidal combination that includes it.

Furthermore, even patients in this trial who were randomized to treatment based on standard AST still received drugs that had showed inhibitory activity *in vitro*. It seems likely that synergy testing may turn out to be most useful not in incrementally improving

outcomes of patients for whom standard therapeutic options exist, but in identifying effective salvage therapy regimens for patients infected with pan-resistant isolates<sup>52,76</sup> for which treatments do not otherwise exist.

#### THE FUTURE OF SYNERGY TESTING

As options for treatment of increasingly resistant bacteria dwindle, synergistic antimicrobial regimens that “rescue” the activity of existing drugs offer the prospect of significantly expanded treatment options. However, both more high-quality data are needed in order to determine which combinations are clinically effective for which organisms and to establish evidence-based standards for performing and interpreting synergy testing in the clinical laboratory. Progress along several axes will be essential to this process.

##### Clinical trials incorporating *in vitro* synergy data

As discussed above, very few clinical trials that compare combination therapy to monotherapy (or different combination regimens to each other) have included *in vitro* synergy testing. Most such clinical studies show mixed outcomes or demonstrate no overall benefit from combination therapy.<sup>9,72</sup> However, if only a subset of bacterial isolates are susceptible to a combination (just as only some isolates are susceptible to any given antibiotic), then these trials, which presumably include patients infected with both synergy-susceptible and synergy-non-susceptible strains with no information about which is which, may show no overall effect even if the combination would have been beneficial for some

patients. Only If future clinical trials test patient isolates for *in vitro* synergy will it be possible to establish a relationship between *in vitro* synergy results and clinical outcomes.

#### *Simpler synergy testing methods*

The technical complexity of synergy testing currently limits its inclusion in clinical trials, particularly in retrospective studies that rely upon data collected during routine clinical care. Furthermore, because on-demand synergy testing is not available clinically for standard bacterial pathogens, the utility of synergy testing-based combination therapy would be limited at present. Reducing the complexity of synergy testing methods, perhaps through automation<sup>27</sup> or the manufacturing of standardized synergy panels, could allow these methods to be more widely adopted in both research and, ultimately, clinical settings.

#### *Maximizing the utility of synergy data*

In addition to simplifying synergy techniques, it will also be important to optimize the utility of the data generated by synergy testing. A great deal of information obtained from synergy studies, particularly time-kill assays and PK/PD models, is not used in traditional synergy definitions but may be of value in establishing clinical efficacy or optimal dosing regimens. For example, if bacterial killing after 6 hours of drug exposure is most predictive of clinical outcome for a certain drug, then an assay could be developed that specifically quantifies killing at 6 hours to simplify testing and improve predictive value. Similarly, data from PK/PD models could be used to optimize dose timing (e.g. administration of synergistic drugs to a patient simultaneously versus at staggered intervals) and to determine whether drugs with

dose-dependent toxicities could be given at lower concentrations if used in combinations, thereby reducing side effects without sacrificing efficacy.

## CONCLUSION

The utility of combination antimicrobial therapy is evidenced by the ubiquity and efficacy of commonly used antibiotic combinations and by the recent introduction of broad-spectrum  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations such as ceftazidime-avibactam and meropenem-vaborbactam, which can treat Gram-negative bacteria that contain a *Klebsiella pneumoniae* carbapenemase (KPC) enzyme, one of the most threatening antimicrobial resistance mechanisms known.<sup>77</sup> A host of literature describing combinations with *in vitro* activity against multidrug-resistant bacteria suggests that there are additional combination options within the armamentarium of existing antibiotics that may have utility in the treatment of patients infected with multidrug-resistant bacteria. More data, especially data that includes results of *in vitro* testing and clinical outcomes from the same bacterial isolates, as well as advances in synergy testing methods, are needed to determine which of these combinations will be most effective in combatting multidrug-resistant infections.



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### Figure Legends

Figure 1: Arrangement of the checkerboard array and calculation of the  $FIC_i$ . Antibiotic concentrations are expressed as multiples of the MIC. FIC, fractional inhibitory concentration;  $FIC_i$ , FIC index;  $FIC_A$ , FIC of antibiotic A;  $FIC_B$ , FIC of antibiotic B.

