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When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing

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In this age of emerging antibiotic resistance, limited therapeutic options exist for treating multidrug-resistant organisms. Combination therapy is commonly employed to manage these infections despite little laboratory guidance as to the efficacy of this approach. Synergy testing methods have been used to assess the interaction of antibiotic combinations *in vitro*. This review will discuss the four primary methods used to assess synergy, as well as the data that exist for testing of cystic fibrosis. In the final analysis, this review concludes that there is not enough evidence to endorse synergy testing for routine clinical use.

One of the most important functions of the clinical microbiology laboratory is to provide predictive information regarding the use of antimicrobials for the treatment of infectious diseases. The methods by which the laboratory assesses susceptibility are highly standardized and remarkably reproducible, especially when considering that biological systems are being tested (1). It is this reproducibility that allows laboratories across the street and across the globe to generate comparable results. In order to make accurate predictions about treatment outcome, laboratories must not only have a reproducible and reliable method, but the *in vitro* data generated by these methods must have relevance to the physiologic conditions found within a patient. For most commonly encountered infections, these data exist in the form of pharmacokinetic/pharmacodynamic (PK/PD) analyses, as well as in outcome studies. By considering a combination of *in vitro* data, PK/PD analyses, and outcome studies, interpretive criteria are established and allow the laboratory to report antimicrobial-organism combinations as susceptible, intermediate, or resistant.

The benefit of this susceptibility testing system is that it is a simple and robust method enabling extraordinary degrees of reproducibility, despite being performed by a wide variety of laboratories. However, the greatest strength of this method may also be its greatest weakness. Because this method is so exceedingly simple, it fails to account for factors that are undoubtedly critical for accurate prediction of treatment outcome. For example, current susceptibility methods do not take into account factors such as patient immune system function, site of infection (with a few exceptions), and drug-drug interactions. It is perhaps not surprising that some have found significant limitations in the ability of these methods to predict outcome. In 2002, Rex and Pfaller assessed the predictive value of susceptibility testing and found that the method was approximately 90% accurate in predicting positive outcomes with susceptible results but only 40% accurate in predicting negative outcomes with resistance (2). They refer to this phenomenon as the "90-60" rule, suggesting that 90% of susceptible results predict success, while 60% of resistant results still have successful treatment outcomes. Interestingly, this finding was not specific to any one antimicrobial, organism, or site of infection. There are many possible explanations for the 90-60 rule, one of which is that our methods fail to account for situations in which a patient is receiving more than one antimicrobial. Traditional susceptibility testing relies on the assessment of individual organism-antimicrobial combinations from which some objective measure of antimicrobial activity is derived. What is not considered is how those organisms will respond to therapy when more than one antimicrobial is used. Indeed, some studies estimate that up to 50% of patients with bacteremia, pneumonia, or surgical infection and more than 50% of patients with septic shock in intensive care units are prescribed combinations of antimicrobial therapy (3–5). Our routine susceptibility testing methods fail to account for these dynamic treatment situations. Given the frequency with which combination therapy is employed, a method capable of assessing antimicrobial interaction and activity could be of some value.

Synergy testing is done with sophisticated susceptibility testing techniques that account for combinations of antimicrobials and measure their cumulative efficacy. What follows is a detailed discussion of the methods that exist for assessing antimicrobial synergy. Specifically, the technical aspects of performing synergy testing will be described, as well as the interpretation of the data provided. The review will then discuss the clinical scenario in which synergy testing has most commonly been applied: testing of pathogens isolated from cystic fibrosis (CF) cases. The majority of this discussion will focus on *in vitro* data, and where available, outcome studies will be considered. Regrettably though, very few outcome data are available to support the use of synergy testing to predict which antimicrobial combinations will be most effective. What outcome data are available suggest that synergy testing is no better than conventional testing with respect to predicting patient outcome. In some cases, the literature suggests that it is in fact deleterious to patient care. Of note, a plethora of antimicrobial combinations not in current use have been assessed for synergy. Many of these combinations have not been used for patient care and are therefore outside the scope of this minireview. In addition, the subject of enterococci and high-level aminoglycoside resistance testing will not be discussed. These tests have been shown to predict the presence or absence of synergy but are themselves not synergy tests.

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$$FIC = \left(\frac{MICA_{combination\ A\&B}}{MIC_{agent\ A}}\right) + \left(\frac{MICB_{combination\ A\&B}}{MIC_{agentB}}\right)$$

FIC Value	Interpretation
≤0.5	Synergy
>0.5-1	Additive
1-4	Indifference
>4	Antagonism

Example

Agent	MIC (Alone)	MIC (Combination)
A	16	2
B	8	2

$$FIC = \left(\frac{2}{16}\right) + \left(\frac{2}{8}\right) = 0.375 = Synergy$$

FIG 1 Depiction of fractional inhibitory concentration (FIC) calculation.

SYNERGY TESTING METHODS

The goal of synergy testing is to assess the *in vitro* interaction of antimicrobial combinations to determine whether the effect of the two antimicrobials is greater than the sum of their individual activities. Antimicrobial combinations can act additively, where the cumulative antimicrobial effect is simply the sum total of the two antimicrobials acting together, or they can act synergistically, where the combined activity is greater than the sum of their activities when used individually. Conversely, these methods are also capable of identifying combinations that are antagonistic. There are four primary methods by which synergy can be assessed *in vitro*: the checkerboard method, multiple-combination bactericidal antimicrobial testing (MCBT), Etest, and time-kill curve assays.

CHECKERBOARD METHOD

The checkerboard method assesses the activities of antimicrobial combinations tested at clinically achievable concentrations in serial 2-fold dilutions. The assay combinations are generally designed to include antimicrobials from different classes. The data produced by the checkerboard assay are analyzed in terms of the fractional inhibitory concentration index (FIC) (Fig. 1). The FIC is calculated by comparing the value of the MIC of each agent alone with the combination-derived MIC. Antimicrobial combinations that result in a 4-fold reduction in the MIC compared with the MICs of agents alone are synergistic (FIC $\leq$ 0.5). FICs in the 0.5 to 1.0 range are considered to be nonsynergistic or additive. FICs from 1 to 4 are defined as indifferent, while those of >4 are antagonistic (6). The limitations of this method are that it only tests antimicrobials for a fixed incubation time, it can require a large number of reagents and resources to test different antimicrobial combinations, and it is not capable of testing more than two antimicrobials at a time. In other words, combinations of three and four antimicrobial cannot be tested.

MCBT

The multiple-combination bactericidal test is designed to test combinations of two, three, or four antimicrobials simultaneously and is based on the premise of doing so at pharmacologically allowable blood concentrations. The concentration used in each experiment is defined by what can be achieved in a patient's serum. In contrast to checkerboard synergy testing methods, only fixed concentrations are assessed with MCBT. The primary variable tested is the combination of the antimicrobial tested. These experiments are typically carried out in a series of 96-well microtiter plates that contain the desired antimicrobial combinations and concentrations. These assays are inoculated with the test organism to a final concentration of 5×10^5 CFU/ml and incubated at 35°C for 48 h. At 24 and 48 h, the wells are inspected for turbidity, and wells without visual evidence of growth are subcultured to solid medium and assessed after overnight incubation for 99.9% killing (7).

TIME-KILL ASSAYS

Time-kill assays (TKA) are a derivative of minimum bactericidal concentration (MBC) testing. MBC testing establishes the concentration of antimicrobial that is necessary to kill 99.9% of an inoculum after a 24-h exposure period. This is also the fundamental principle used in MCBT, and its utility is limited because it relies on a somewhat arbitrary (99.9%) definition of complete killing that is tested in a single, fixed time point. Furthermore, some have questioned the utility of MCBTs because of the highly technical nature of the testing and the difficulty in controlling variables (8). TKAs take the principle of the MCBT, but rather than assessing cidal activity at a single point in time, the activity is assessed in a chronological fashion over a 48-h incubation period. In contrast to the checkerboard assay, which assesses killing at a fixed time point and establishes the optimal concentration for killing, TKAs determine the rate of killing, which may be a more relevant metric to predicting patient outcome (9).

In 1999, the National Committee on Clinical Laboratory Standards (NCCLS; now CLSI) published a document which provided guidance for bactericidal testing. Within that document, a standardized protocol for time-kill assays is provided (10). Briefly, TKAs are done in large volumes (>10 ml) in glass beakers where the bacterial inoculum is placed into broth containing the desired concentration of antimicrobials. The inoculum is then incubated for a total of 48 h, with periodic 0.5-ml aliquots being collected and plated for colony count determination. These samplings generally occur at 4, 8, 10, 12, and 24 h. The time-kill colony counts are then graphically represented as a function of time. Synergy for time-kill assays is then defined as a >2 $\log_{10}$ increase between the results for the antimicrobial combination and the results for its most active constituent (10). Interpretation of TKAs can be difficult due to bacterial regrowth at later time points, and they are best assessed in the first 12 h.

SYNERGY BY ETEST

Conventional, single-drug testing with the Etest (otherwise known as gradient diffusion) relies on the diffusion of a continuous concentration gradient of antimicrobial from an impregnated strip into solid agar. Etest strips are placed on agar medium that has been inoculated with a lawn of the test organism. The Etest is then incubated overnight, and the point at which the elliptical no-growth zone touches the strips can be read as the MIC. There are two modifications of this procedure which have been developed to assess synergy. In the first method, two Etest strips, each containing one of the antimicrobials of interest, are placed per-

pendicular to each other, intersecting at the MIC for each antimicrobial when tested alone. As with the checkerboard technique, the interpretation of Etest synergy is based on the FIC calculation, which is presented in Fig. 1 (6, 11). A second approach to Etest synergy places the first Etest strip (containing drug number 1) on the agar which has been inoculated with a lawn of the test organism. The Etest is allowed to sit for 60 min and is then removed. A second strip (containing drug number 2) is then placed in the same position. This represents the synergy portion of the assay. Control Etests for each individual drug are placed on the same plate such that they do not interfere with the synergy test. Using this method, synergy is defined as a $\geq$3 dilution decrease in MIC, additivity as a decrease of $\geq$2 but $<$3 dilutions, and indifference as a decrease of $<$2 dilutions in the MIC. Antagonism is defined as an increase of $\geq$3 dilutions of the MIC (12).

COMPARISON OF SYNERGY TESTING METHODS

With four different approaches to synergy testing, a number of studies have endeavored to compare their results in an effort to identify the best method. One challenge in doing so is that there is no true gold standard for synergy, so it is difficult to know which results are correct. Nonetheless, Lewis and colleagues were able to conclude that the checkerboard method was inferior to Etest and TKAs when assessing antifungal synergy for *Candida* species (12). This finding may be due to the MIC clustering that broth microdilution can demonstrate in antifungal testing. This phenomenon may make it difficult to discriminate between susceptible and intermediate isolates and, therefore, hinder the performance of checkerboard testing. For bacterial testing, White and colleagues compared the TKA, checkerboard, and Etest methodologies for *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. For these experiments, they considered TKA to be the gold standard and found that agreement ranged from 44 to 88% and 63 to 75% for the checkerboard and Etest methods, respectively (11). The authors conclude that this is a positive finding and, given, the relative simplicity of the Etest method, suggest that it could be a viable alternative to TKA and checkerboard testing. However, if we accept that TKA is the gold standard for assessing synergy, then it is difficult to see the Etest as an acceptable method in the clinical laboratory with up to one quarter of results being discrepant and potentially erroneous.

Several other studies have evaluated the comparability of synergy testing and found various degrees of agreement. Cappelletty and Rybak compared checkerboard to TKA for *Pseudomonas aeruginosa* and found almost no correlation between the two methods. Their primary finding was that TKA demonstrated consistent synergism at various concentrations of antimicrobial, while checkerboard testing could only demonstrate indifference (13). These two studies reflect the consensus of others who have compared synergy methods for different antimicrobial combinations and bacterial species and all found the same thing, that no two synergy methods produce comparable results (14–17). However, in some circumstances, $>$90% correlation between Etest and checkerboard testing has been observed (18).

CLINICAL APPLICATION OF SYNERGY TESTING

As was just discussed, the literature suggests that no two synergy methods produce comparable results. It is therefore unlikely that, as a whole, synergy testing will prove to be clinically relevant. However, it is possible that one of these methods produces results that correlate with patient outcomes. As was stated earlier, there is no true gold standard for synergy testing. TKA appears to be the primary comparator most commonly used in the literature, which is likely due to the fact that NCCLS standardized the method. This makes some sense, as the method produces dynamic information about organism killing over time, something not provided by other methods. However, only a few studies have attempted to establish the true clinical relevance of synergy testing through outcome-based studies, and none of these evaluate TKA. The result is that a tremendous amount of *in vitro* data analyzing synergistic antimicrobial combinations are available, but almost none of that information can be linked to treatment outcomes.

Despite the paucity of outcome data, synergy testing has been used to predict patient outcomes in cystic fibrosis patients. The following sections will review what is known about the synergy testing in this patient population and discuss the applicable outcome studies.

CYSTIC FIBROSIS AND SYNERGY TESTING

Cystic fibrosis (CF) patients may be at greater risk for developing multidrug-resistant infections than any other patient population (19). Organisms such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia* are common pathogens in these patients and are often extremely resistant, leaving few or no therapeutic options. Initially, cystic fibrosis patients seemed like ideal candidates for synergy testing. Indeed, until 2009, the Cystic Fibrosis Foundation had recommended that synergy testing be considered in CF patients with multidrug-resistant organisms. The CF Referral Center at Columbia University was established in 1992 to satisfy the growing demand for synergy testing (6).

The literature shows that all of the four methods discussed above have been applied to cystic fibrosis isolates. However, the two methods most commonly used have been the checkerboard method and the MCBT method. Taken together, it is difficult to make any general conclusions from this body of literature, though perhaps the disparity of findings is by its nature informative. It is clear that broad statements about synergistic combinations for specific organisms cannot be made. If synergy testing is to be useful clinically, patient isolates would require testing on a case-by-case basis.

The literature has primarily focused on multidrug-resistant organisms and has come to a variety of conclusions. Lang and colleagues looked at 75 CF strains from 44 patients using the MCBT method. After evaluating an average of 11.5 drug combinations per isolate, they found meropenem-based regimens to be the most synergistic, with meropenem plus ciprofloxacin exhibiting activity against 85% of strains (20, 21). This excludes the combination of meropenem plus high-dose tobramycin, which was active against 94% of isolates. Other studies have focused on specific pathogens, such as *Stenotrophomonas maltophilia*, where the checkerboard method demonstrated that 65% of 673 CF-isolated strains were inhibited by a combination of trimethoprim-sulfamethoxazole plus ticarcillin-clavulanate with either a synergistic or additive effect (22). Another important CF pathogen, *Burkholderia cepacia*, was evaluated by the same group using checkerboard testing. Of 2,621 strains from 1,257 patients, synergy was only identified in 1 to 15% of strains (23). Aaron and colleagues tested a different subset of *Burkholderia cepacia* isolates using the

MCBT method and found that triple antimicrobial combinations were bactericidal in 81 to 93% of isolates, which was superior to double antimicrobial combinations (7). While these results are not comparable because the methods used produce different types of information, it is remarkable how different the findings appear to be despite the fact that they were testing the same organism from similar patient populations.

Without question, the best outcome study to look at the efficacy of synergy testing was conducted by Aaron and colleagues in the cystic fibrosis patient population (24). In this study, 251 patient with cystic fibrosis were enrolled into a randomized, double-blind, controlled trial. Patients developing an exacerbation received a 14-day course of one of two blinded intravenous antimicrobial regimens. One was chosen on the basis of conventional susceptibility testing, and the other was selected based on synergy testing conducted by the MCBT method. The study lasted 4.5 years and ultimately included 132 patients. In the final analysis, the authors concluded that there was no difference between the groups in treatment failure rate, changes in lung function, dyspnea, or bacterial density. Based almost entirely on these findings, the 2009 Cystic Fibrosis Foundation practice guidelines specifically stated that synergy testing should not be done in cystic fibrosis patients (25). This sentiment is certainly supported by a 2007 review claiming that synergy testing should not be performed in cystic fibrosis patients (26). This conclusion is based exclusively on the results found in the MCBT-based outcome study discussed above. While this was a high-quality study and the results convincing, it is important to note that no other method of synergy testing was assessed and conclusions about those methods cannot be drawn. It is possible that methods such as the checkerboard technique or TKA may offer a more clinically relevant option. At present, though, no outcome data exist to make a strong statement about the clinical utility of these alternative methods.

SUMMARY

In conclusion, there are a number of options that exist for assessing combinations of antimicrobials for synergism. The methods vary widely in their complexity and interpretation. This variation probably explains the significant lack of correlation observed in head-to-head comparison studies.

Based on the literature presented in this review, one can conclude that synergy testing (regardless of method) cannot be recommended for routine use to guide patient care. The reasons for this are 2-fold. First, the term "synergy test" actually refers to several different methods (TKA, MCBT, Etest, and checkerboard) that all produce different results. In the absence of a true gold standard, it is unknown which method would produce the most accurate and clinically relevant results. Second, although several synergy testing methods incorporate physiological concentrations of antimicrobials, nearly all data have been generated through *in vitro* experiments. While interesting, these data do not inform as to the clinical outcomes that correlate with these methods. Only one clinical outcome study has been conducted (using MCBT), and it failed to show that synergy testing could be used to improve patient outcomes (24). In fact, the only statistically significant finding was that the patients' mean hospital length of stay was 3 days longer in the MCBT intervention group ($P = 0.03$). Unfortunately, this is the only study that speaks to the utility of synergy testing in patient care. The fact that there is very little correlation between the results of different synergy methods may mean that another method may better predict patient outcomes. At this time, we await outcome studies evaluating these other methodologies. Such studies would be needed before other methodologies could be endorsed as part of the routine clinical workflow.

And finally, a significant limitation of these methods is that they are complex, labor intensive, and require detailed understanding for interpretation. Etest synergy methods offer a simplified work flow, but their results have not been found to correlate with those of better-established methods, such as TKA.

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