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Methodological Variation in Antibiotic Synergy Tests Against Enterococci

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Thirty-two human isolates of enterococci were tested for antibiotic synergy by using penicillin and one of six aminoglycosides. Three methods were used: synergy screen, microdilution checkerboard, and time-kill curves. The synergy screen accurately predicted synergy for gentamicin-penicillin combinations, and this synergy was later confirmed by time-kill curves. The microdilution checkerboard method suffered from inherent variation, and agreement with time-kill curves ranged from 92% (twofold reduction in minimum inhibitory concentration) to 4.2% (fourfold reduction in minimum inhibitory concentration). We suggest that enterococci be screened for synergy (i.e., presence or absence of high-level resistance) by using the criterion of growth or no growth in the presence of 2,000 μg of an aminoglycoside per ml. The microdilution checkerboard test for synergy is not recommended.

Enterococcal infections, especially endocarditis, are often treated with two antibiotics, which results in higher cure rates than when one agent is used alone. The therapeutic combination usually includes a penicillin and an aminoglycoside (5). The rationale for this approach depends on (i) the ability of the cell wall-active antibiotic to modify the architecture of the cell wall and increase permeability of the aminoglycoside antibiotic, (ii) the susceptibility of the ribosomes to attachment and inhibition by the aminoglycoside, and (iii) the presence of aminoglycoside-inactivating enzymes. Several methods have been proposed to predict antibiotic synergy. They include the time-kill curve (TKC) (5), the microdilution checkerboard (MDC) method, and the synergy screen (SS) for ribosomal susceptibility (2).

Norden et al. (7) have compared synergy techniques for *Klebsiella* by using cephalothin and gentamicin. There was poor correlation between the MDC and TKC results. Weinstein et al. (8) published a similar report on *Pseudomonas* and *Serratia* and showed excellent correlation between analytical methods. The present study investigated the relationship of the SS for high-level resistance, the MDC, and the TKC for the prediction of synergy. Thirty-two clinical isolates of enterococci were tested, using penicillin in combination with one of six aminoglycosides (gentamicin, tobramycin, kanamycin, amikacin, streptomycin, and netilmicin).

MATERIALS AND METHODS

Isolates. Thirty-two strains of enterococci isolated from clinical specimens (blood, urine, and wounds) at the John Dempsey Hospital of the University of Connecticut Health Center were included in the study. Enterococci were identified by accepted methods and maintained in Trypticase soy broth (BBL Microbiology Systems) with 15% glycerol at − 20°C until tested. Before testing, the isolates were again subcultured on 10% sheep blood agar and examined for purity. Single colonies were transferred to 5 ml of Mueller-Hinton broth and incubated at 35°C for 18 to 24 h. All inocula were standardized at 1.5×10^7 to 3.0×10^7 colony-forming units per milliliter by using the Autobac light-scattering photometer (Pfizer Diagnostics, Inc., Groton, Conn.). Dilutions of the standardized inocula were made as required.

Antibiotics. All antibiotics used were USP reference standards. Aqueous solutions were freshly prepared before use and never frozen for subsequent use.

Synergy screen. The method of Calderwood et al. (2) was used to detect high-level resistance to aminoglycosides. Isolates were inoculated with a Steers-Foltz replicator on a Mueller-Hinton agar plate containing 2,000 μg of aminoglycoside per ml. Plates were incubated at 35°C for 18 to 24 h, and results were recorded as growth (high-level resistance) or no growth (high-level susceptibility).

MDC. The MDC method of Bourque et al. (1) was used to predict synergy. Twofold dilutions of one drug were tested in combination with twofold dilutions of the other. The inhibitory endpoint was that well containing no visible turbidity or particulate growth. Each strain was tested at least twice by the microdilution method.

TKCs. Tests for synergy were performed in Mueller-Hinton broth, using the growth curve method of Moellering et al. (5). An overnight culture of the test strain was adjusted to an initial concentration of 1×10^8 to 5×10^8 colony-forming units per ml. Antibiotics were added as follows: penicillin G, 10 U/ml; gentamicin, either 2.5 or 5.0 μg/ml but always less than the minimum inhibitory concentration (MIC) for genta-

micin. The assay tubes were incubated at 35°C and sampled at 0, 4, and 24 h, and standard plate counts were performed.

Criteria for synergy. Criteria for synergy were as follows: SS—no growth in the presence of 2,000 μg of aminoglycoside per ml indicated potential synergy; MDC—a reduction in MIC of both antibiotics of at least two dilution intervals (fourfold); TKCs—a 100× reduction in bacterial colony count in the presence of both antibiotics as opposed to each one individually.

RESULTS

Thirty-two isolates of enterococci were evaluated by SS and MDC methods for synergistic killing by using penicillin and each of six aminoglycosides: gentamicin, kanamycin, tobramycin, streptomycin, amikacin, and netilmicin. The same 32 isolates were evaluated using TKCs with gentamicin and penicillin. Of the six aminoglycosides tested by SS, only two, kanamycin and streptomycin, showed high-level resistance in 28% (9/32) and 33% (11/32) of the isolates, respectively.

Table 1 shows the correlation of the SS with the MDC method for the six antibiotic pairs and 32 isolates. If the twofold reduction of both penicillin and aminoglycoside MIC was accepted, then the range of agreement with SS was from 85.2% (amikacin-penicillin) to 95.0% (kanamycin-penicillin). If a fourfold reduction in the MDC MICs was used, then agreement ranged from 3.7% (gentamicin-penicillin) to 18.5% (netilmicin-penicillin). With those aminoglycosides (kanamycin-streptomycin) in which the SS predicted "no synergy," agreement was 85.7% for both kanamycin-penicillin and streptomycin-penicillin.

For the gentamicin-penicillin combination, there was 89% agreement between SS and TKC, 92% agreement between MDC (twofold reduction) and TKC, and 4.2% agreement between MDC (fourfold reduction) and TKC.

TABLE 1. *Agreement between the SS and MDC methods for penicillin-aminoglycoside synergy*

Antibiotic combination	Agreement, MDC/SS (%)		
	2×[a] synergy	4×[a] synergy	No synergy
Amikacin-penicillin	85.2	7.4	—[b]
Gentamicin-penicillin	92.6	3.7	—
Kanamycin-penicillin	95.0	10.0	85.7
Netilmicin-penicillin	88.9	18.5	—
Streptomycin-penicillin	95.0	5.0	85.7
Tobramycin-penicillin	88.9	7.4	—

[a] Indicates at least twofold or fourfold reduction in the MIC of both antibiotics.

[b] —, Synergy predicted in 100% of the isolates.

Figure 1 represents 1 of 32 human enterococcal isolates studied by the TKC method using a gentamicin-penicillin combination. There was approximately a 100× reduction in colony count with the penicillin-gentamicin combination compared to either drug alone. When all TKCs were evaluated (32 isolates), a 10× reduction in colony count of the combination versus the individual antibiotics resulted in 100% synergy, and a 100× reduction resulted in 60% synergy.

DISCUSSION

Norden et al. (7) found that the frequency of synergy for 22 isolates of *Klebsiella* varied significantly with the method. This report presents similar data for 32 strains of enterococci. Although methods for synergy analysis are not standardized from laboratory to laboratory, care was taken to insure "within-laboratory" standardization of media, inocula, time and temperature of incubation, and reading of results. The data suggest that even if methods were standardized, difficulties still might exist, such as interpretation of results, setting of synergy criteria, and methodological and biological variation.

The correlation of SS and TKC was acceptable for gentamicin-penicillin (89%). SS is a yes/no test. If the isolate fails to grow in the presence of 2,000 μg of gentamicin per ml, bacterial ribosomes are presumably susceptible to the bactericidal effects of gentamicin. If cell wall integrity is destroyed by a cell wall-active agent, the lesser concentrations of aminoglycoside should effectively bind 30S portions of the affected ribosomes. The interpretive agreement between this screening test and TKC is not surprising since

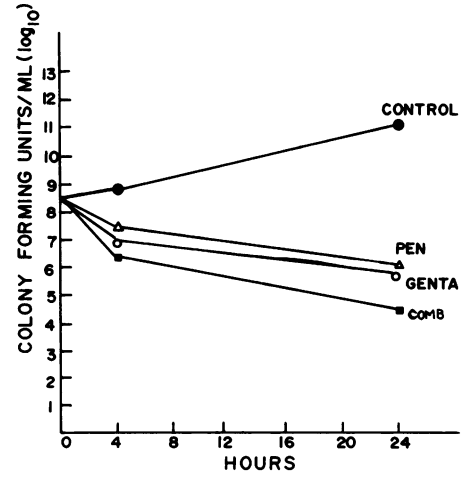

FIG. 1. *A representative enterococcus TKC for gentamicin and penicillin alone and in combination.*

both assays measure cell killing and not only inactivation. However, the extent of agreement between SS and TKC is a function of the rigor with which synergy is defined in the TKC method. Using the criterion of 10× reduction in colony-forming units per milliliter in the presence of both antibiotics, 100% of the isolates showed synergy. However, if the stricter criterion of 100× reduction in colony-forming units per milliliter was used, then only 60% (19/32) of the isolates showed synergy.

The agreement between SS and MDC for the six antibiotic pairs and both TKC and MDC for gentamicin-penicillin was also a function of the rigor with which MDC synergy was defined. If a fourfold reduction in the MICs of both drugs in combination was required for synergy, then agreement with the other two methods was poor. If a twofold reduction was used, then agreement was >90%. However, the MDC method, as done in most laboratories, uses microtiter loops for the twofold dilution steps. It is universally recognized that the minimum error in any serial twofold dilution is one dilution interval. Thus, in the MDC, rarely was a fourfold reduction in both MICs seen when synergy was detected by other methods, and a twofold reduction was within the error of the method.

The MDC assay defines an inhibitory endpoint, not a bactericidal one. In this study, the MDC bactericidal endpoint was not determined. Although, when testing the enterococci, the MIC usually equals the MBC for bactericidal drugs, this correlation cannot be assumed.

This study showed that the MDC assay is not a reliable predictor of synergy when inherent test variation is considered. Furthermore, growth of enterococci in Mueller-Hinton broth is often difficult to read, and bacteriostatic endpoints may be obscured. We cannot recommend MDC as a routine procedure. Possibly, if MDC trays are made with a dispensing instrument that does not rely on serial dilution, then test error can be minimized. The data suggest that the SS may be applicable for all aminoglycoside antibiotics, with the possible exception of amikacin. TKCs, however, were performed on only the most widely used antibiotic pair, gentamicin and penicillin.

In this study, there was no species differentiation between *Streptococcus faecalis* and *Streptococcus faecium*. Moellering et al. (6) have reported that *S. faecium* strains are often resistant to penicillin-tobramycin synergism, although all strains of *S. faecalis* examined were susceptible. The present study failed to identify penicillin-tobramycin resistance in any of the 32 enterococci tested.

Calderwood et al. (2) indicated that 40 to 50% of clinical isolates of enterococci showed high-level resistance to both kanamycin and streptomycin and resistance to penicillin-kanamycin and penicillin-streptomycin synergy. We have noted that 28% (9/32) and 33% (11/32) of the isolates showed resistance to kanamycin and streptomycin, respectively. Although no high-level resistance was observed with amikacin, Calderwood et al. (2) observed that only 1 of 10 enterococcal strains showed high-level amikacin resistance, but 6 of 10 were resistant to synergistic killing by the penicillin-amikacin combination. The relatively low agreement (85.2%) noted for amikacin-penicillin between the MDC synergy test and the SS may in part be a reflection of this phenomenon.

Laboratory evaluation of antibiotic synergy appears to be important as there is evidence that patients treated with synergistic combinations of antibiotics have lower mortality than those receiving nonsynergistic combinations (3, 4). Consequently, precise and accurate predictors of synergy must be standardized to reduce in-laboratory variation and to provide reproducible inter-laboratory data.

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