A SEMI-AUTOMATED METHOD FOR DETERMINING THE IN VITRO ACTION OF ANTIBIOTICS IN COMBINATION, WITH A SURVEY OF VANCOMYCIN AND THE AMINOGLYCOSIDE ANTIBIOTICS AGAINST CLINICAL ISOLATES OF ENTEROCOCCI.

A Thesis
Presented in Partial Fulfillment of the Requirements for the Degree Master of Science

by

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1975

Approved by

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Advisor
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Acknowledgement

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I also am grateful to the staff of the clinical bacteriology laboratory for aid in obtaining organisms used in this study.

In addition, I wish to extend to my parents my deepest gratitude for their belief in my goals and their unending encouragement in attaining them.

This work is dedicated to the prime mover in my life, my wife Vicki, who was a constant source of encouragement, motivation, and understanding, as well as typist.
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INTRODUCTION

Enrich summarized the principles of chemotherapy as the use of a specific "magic bullet" for the elimination of a specific infectious agent. This philosophy has served well with certain diseases, such as pneumococcal pneumonia, syphilis, B-streptococcal pharyngitis and penicillin. However, with some infectious agents, two "magic bullets" aimed together, produce more gratifying results. Synergy has been defined as the use of two antibiotics to obtain better results than obtained by either antibiotic alone or by additive effects of the two combined.

Subacute bacterial endocarditis (SBE) due to enterococcal species is an example of this need for synergy. Historically, SBE has been an almost universally fatal disease, recovery being a rare event (37) in the pre-antibiotic era. The discovery of sulfonamides resulted in a recovery rate of about 10% (36), and later, the use of massive doses of penicillin raised the rate to nearly 80% (27). The introduction of combination therapy (two "magic bullets") with the addition of streptomycin in 1947 by Hunter (28) increased treatment effectiveness to greater than 90% recovery (34, 56, 66) and has been the recommended treatment for the last 15 years (2). Enterococci have become increasingly resistant to most antibiotics at achievable serum levels (63, 65) during this period of time, and a significant number of strains (reports vary from 30 to 80% [53]) are in fact
now resistant to the penicillin - streptomycin regimen. Other aminoglycosides, including gentamicin and tobramycin, have been tested with penicillin for synergism. Penicillin in combination with gentamicin has shown promise (51), and is now the preferred combination for treatment of enterococcal endocarditis at Massachusetts General Hospital in Boston.

There is still no accepted antibiotic combination for those patients who are allergic to penicillin, or who for other reasons are unable to tolerate an extended penicillin - streptomycin course of therapy. For these patients, various alternatives have been proposed: administration of antimistimulins prior to penicillin; cephalosporins alone or in conjunction with streptomycin; and even penicillin alone, administered slowly with close observation of the patient for onset of hypersensitivity symptoms (61). Vancomycin, an inhibitor of cell wall synthesis somewhat similar to penicillin (61), is markedly inhibitory for the enterococci, but is bactericidal only in concentrations far greater than those clinically achievable (23). There are reports of ME being treated with vancomycin alone (15), and recent work has shown that, in combination with streptomycin or gentamicin, synergy can be obtained in vitro (23, 44, 71, 75).

The objective of this study is the development of a semi-automated method for performing in vitro synergy studies for two antibiotics in combination. This method will then be used to study vancomycin
in combination with the newer aminoglycoside antibiotics against clinical isolates of enterococci at clinically achievable concentrations to determine patterns of synergy.
LITERATURE REVIEW

Ungar's (67) observation in 1943 that penicillin in the presence of sulfonamides exerts a synergistic effect on certain bacteria stimulated considerable interest in the possibility of other combinations being successfully applied to those bacteria unsuccessfully eradicated by a single drug regime.

In 1947, Buntre (28), in a clinical study of several patients with enterococcal endocarditis, demonstrated the efficacy of using penicillin in combination with streptomycin over the use of either antibiotic alone. Robbins (96), in 1949, noted that three patients were cured following a 4-8 week regimen of moderate doses of both penicillin and streptomycin, and in the same year, ramputt (66) reported the clinical value of this combination. Since that time numerous publications (17, 18, 22, 35, 37, 38, 39, 70) have confirmed the value of penicillin in combination with streptomycin for treatment of enterococcal endocarditis.

In 1952, Jewett and Gunnison (33) presented a classic paper concerning synergism and antagonism between certain antibiotics. According to their scheme (Fig. 1), there are four groups of antibiotics: groups I and II are bactericidal; groups III and IV are bacteriostatic. The interactions between the various groups are a function of the mode of action of the particular antibiotics.
Fig. 1. Interactions between various antibiotics, grouped according to mode of action.
Summarized, synergy could be expected from combining two bactericidal antibiotics; whereas other combinations resulted in indifference, or even antagonism. In 1960, Minton and White (45) modified this scheme: a) as a rule, antibiotics from the same group can be combined with no antagonism; b) antagonism is only rarely encountered when group I antibiotics are combined with any of the other three groups, and may even yield synergetic results (v.l., penicillin plus streptomycin); c) group II plus group III often results in antagonism since the bacteriostatic antibiotic will predominate; and d) group II plus group IV yields indifference, or rarely synergy (v.l., penicillin plus sulfonamides).

Note: (30), in 1968, noted that occasional strains of Staphylococci demonstrated total resistance to penicillin in combination with streptomycin, contrary to current thought which held all strains to be susceptible. Treatment failures resulting in death pointed to the need for new approaches. One suggested regimen involved replacement of streptomycin with another aminoglycoside, kanamycin. Hewitt et al. (26) had shown in 1966 kanamycin to be more effective than streptomycin in vitro, on a weight-to-weight basis. Also suggested was that the mechanism of synergy lay in the ability of penicillin to induce L-forms which were then acceptable to the actions of the aminoglycoside at the cell membrane.
Friedberg et al. (15) in 1960 advocated the alternative of administering vancomycin both to those patients with strains resistant to penicillin plus streptomycin, and to those who were allergic or intolerant to the combination for any extended period. The four patients prevented were cured by 2-3 g IV/day over 4-6 weeks, but skin rashes, fever, and phlebitis at the site of injection were commonly observed side-effects. Moreover, ototoxicity occurred in cases where renal failure lead to unusually high blood concentrations of vancomycin.

Wilkuske et al. (76) in 1970 tested 25 strains of enterococci isolated from endocarditis patients in vitro with penicillin or ampicillin in combination with streptomycin, kanamycin, or gentamicin. Vancomycin plus streptomycin was also tested. Following overnight culture in tryptic soy broth (TSB), 1 ml of a 10^-3 dilution (3.7 x 10^5 organisms) was added to 1 ml TSB containing graded concentrations of antibiotic combinations in a two-dimensional checkerboard arrangement. Following 18-24 hr. incubation, tubes showing no growth and 0.05 ml transferred to 10 ml thioglycolate broth. The thioglycollate tubes were incubated for 48 hr., and the tube having the lowest concentration of antibiotics and showing no growth was called the minimal bactericidal concentration (MBC). Synergy was considered established if the MBC of each drug in combination was less than or equal to 1/4 the MBC of that antibiotic alone. Of 23 strains tested, 11 were resistant to penicillin plus
streptomycin, as were 7 with the vancomycin-streptomycin combination. Results also indicated a distinction between group D enterococcal and group D non-enterococcal species, since the non-enterococcal species were uniformly susceptible to low concentrations of penicillin alone and all the enterococci were resistant.

Standiford et al (62) in 1970 proposed that the susceptibility of enterococci to penicillin-streptomycin or penicillin-vancomycin combinations could be predicted by a simplified minimal inhibitory concentration (MIC) test. Data suggested a natural break-point in the aminoglycoside MIC's between synergistic and non-synergistic enterococci. The streptomycin break-point was approximately 2000 μg/ml; and vancomycin, approximately 3750 μg/ml. Both antibiotics were diluted to these concentrations in trypticase soy yeast (TSY) broth and inoculated with 10^8 colony forming units (CFU)/ml of enterococci in TSY. Following 18-24 hr. incubation, no growth in the tube was interpreted to indicate probable synergistic results using that antibiotic in combination with penicillin, whereas growth indicated probable resistance to the combination. Of the 30 strains synergistic to penicillin plus streptomycin by the conventional tube method, 27 were found to be susceptible to the streptomycin simplified MIC test. Concurrently, Kellerman et al (51) conducted a similar study with the addition of gentamicin. The simplified MIC test was modified by incorporation of 2000 μg/ml of the aminoglycoside into enriched nutrient agar plates (dextrose-yeast extract broth with 0.7%
agar). Strains to be tested were streaked on the plates and incubated overnight. Growth indicated high-level aminglycoside resistance. Conventional tube dilution studies were run in parallel, using a two-dimensional checkerboard arrangement of tubes containing antibiotics in phosphate-dextrose broth. One ml of overnight culture was added (approximately 10^7 CFU/ml), and the tubes were incubated for 18-24 hr. Growth-curve assays were constructed by removing 0.05 ml at discrete intervals, serially diluting in enriched nutrient agar, and making pour-plates which were counted after 18-24 hr. Incubation. Synergy was defined as a 100-fold decrease in CFU/ml by antibiotics combined in comparison to the decrease found with single antibiotics. Results indicated that of the 16 strains susceptible to high levels of streptomycin, 15 showed synergism; as did 19 of 22 strains susceptible to high levels of tetracycline, and 28 of 30 susceptible to high levels of gentamicin. Unfortunately, some strains resistant to high levels of aminglycosides demonstrated synergy in the presence of penicillin while several strains susceptible to high levels showed no synergy. The results indicated that simplified MIC testing cannot replace the more cumbersome tube dilution techniques.

Mandell et al (44) in 1970, using Jouve and Newton's tests, tested the in vitro efficacy of vancomycin, a bactericidal antibiotic which inhibits cell wall synthesis, in combination with streptomycin against S. aureus. Seven dilutions of antibiotics, both alone
and in combination, were made in trypticase soy broth (TSB),
and incubated with $10^8$ CFU/ml from an overnight culture in TSB.
From these, growth curve assays were performed. Results indicated
that of 20 strains tested, 18 were susceptible to 2.5 µg/ml
vancomycin plus 12.5 µg/ml streptomycin. Although these 18 strains
were called synergistic in their response, no definition of
synergism was stated.

The mechanism of synergy became better understood in 1971
when Zimmermann et al. (76) showed that there were two levels of
enterococcal resistance to the aminoglycosides. The first was a
moderately high level of resistance common in most isolates; the
second was a very high level found only in the non-synergistic
strains. The moderately high-level resistance was a permeability
phenomenon, overcome by simultaneous exposure to a cell wall
inhibitor (penicillin or vancomycin). The very high-level resistance
was thought to be a property of the enterococcal ribosome. Møller-Jørgensen
and Weisberg (79) pursued the problem, using $^{14}C$-labeled strepto-
mycin to monitor the uptake mechanisms. No evidence was found to
verify earlier speculation of induction of $I$-forms by penicillin
and subsequent killing by the aminoglycoside. Rather, penicillin
destroyed the cell's impermeability to the aminoglycoside with
subsequent uptake and cell disruption. The exceptions were the
non-synergistic strains in which uptake of aminoglycoside, for
reasons not understood, failed to cause ribosomal reading errors
in protein synthesis.

Concurrently, Watanakunakorn (70) tested 30 strains of enterococci against combinations of penicillin and streptomycin or genta-
micin. Five concentrations of these antibiotics alone and in combination were diluted in 9 ml TSB and inoculated with 1 ml of
overnight growth in TSB diluted to contain between $6 \times 10^5$ and
$6 \times 10^6$ CFU/ml. Growth curve assays were performed, and synergy was
defined to be at least a 100-fold decrease in CFU/ml in comparison
to penicillin alone. A combination of 20 μg/ml penicillin and 4 μg/ml
gentamicin inhibited all 33 strains, whereas 20 μg/ml penicillin
plus 20 μg/ml streptomycin inhibited only 20 strains. Of these 20,
4 were highly resistant to streptomycin by the method of Standiford
et al.

Weinstein and Hoellering (73) in 1973, based upon their earlier
in vitro work, successfully treated 6 cases of enterococcal endocar-
ditis with a combination of penicillin and gentamicin. Two of these
patients were found to have strains highly resistant to streptomycin.

Westenfelder et al (75) in 1973 published the first report of
a patient cured of enterococcal endocarditis by a combination of
vancomycin (500 mg IV/8hr) and streptomycin (1.0 gm/day), in whom
hypersensitivity precluded the use of penicillin. Included was an
in vitro study of 18 enterococcal isolates comparing streptomycin
in combination with penicillin and vancomycin. Several concentrations
of each combination were diluted in 10 ml TSB, and 0.1 ml of
1:10 dilution of overnight growth in TSB was added for an inoculum
of between 10⁵ and 10⁶ CFU/ml. After 24 hrs incubation, when showing
no growth had a loopful of broth (0.01 ml) subcultured to blood agar
plates which were incubated another 24 hrs. Synergy was defined
to be a 1/4 or more reduction in the MBC of antibiotics in combina-
tion as compared to the MBC of each antibiotic alone. Twelve of
the 18 strains showed synergism to vancomycin plus streptomycin,
and 9 of 18 to penicillin plus streptomycin.

Wunnakunakorn and Baktie (71) in 1973 published a synergy study
of 41 enterococcal isolates against vancomycin and streptomycin or
gentamicin using the same methods as in earlier work. Results
demonstrated 10 µg/ml vancomycin plus 10 µg/ml streptomycin to be
synergistic against 29 of the 41 strains, whereas the same amount
of vancomycin and 4 µg/ml gentamicin were synergistic for all 41.
Concurrently, Horwitz et al (23) performed similar studies using a
manual micro-dilution method. Two sets of dilutions, 1 per antibiotic,
were carried out in a 96-well Microtiter plate in a checkerboard
arrangement. Each well contained 0.15 ml Mueller-Hinton broth (M)
and antibiotics, to which 0.05 ml of a 1:200 dilution of overnight
culture (approximately 10⁵ CFU/ml) was added. Synergy was defined
as reduction in the MBC of both antibiotics by a doubling dilution
factor of two or more. Of 36 strains, 33 were susceptible to
6 µg/ml vancomycin and 26 µg/ml streptomycin, and 31 were susceptible
to 6 μg/ml vancomycin plus 12 μg/ml gentamicin.

Brellinger et al. (53) in 1973 compared penicillin plus genta-
icin against penicillin plus tetracycline using methods described
earlier. Of 27 strains of enterococci, all were susceptible to
10 μg/ml penicillin and 5 μg/ml gentamicin, but only 23 showed
synergy when tetracycline was substituted in the same concentra-
tion. Further investigation revealed the 4 non-synergistic strains to be
S. faecium, a separate species within the enterococcal classification.

Harwick et al. (24) in 1974 continued earlier work by studying
in vivo effects of administering either streptomycin or gentamicin
combined with vancomycin to rats with enterococcal pyelonephritis.
Earlier in vitro data favoring the vancomycin-gentamicin combination
were confirmed.

Few attempts have been made to alter the standard tube dilution
technique. Elie and Nilson (10) in 1953 impregnated filter paper
discs with antibiotic and placed the discs on agar plates seeded
with the test organism. The discs were placed in an L-configuration
equidistant from one another, the "ends" being different antibiotics
and the "angle" composed of one disc of each superimposed. Follow-
ing overnight incubation, a sterile velvet pad was used to replicate
the seed plate onto an unenriched agar plate which was incubated
another 18-24 hours. Growth patterns around the discs were used
to qualitatively state presence or absence of synergism. Chobbert (6)
in 1957 modified the technique by substituting filter paper strips
impregnated with antibiotic, and a cellophane disc the size of the
plates to replicate the seed plate. Interpretation of growth
patterns was similar. In 1965 Chabbert (?) reported that the
method was subject to antibiotic carryover and was, at best, of
questionable validity.
MATERIALS AND METHODS

MATERIALS

I. Equipment

A semiautomated microdilutor (Cassico, Inc., Fig. 2) was used to make test plates for both the MIC-MBC determinations of single and combined antibiotics. The Autotiter III consists of a movable carriage upon which are mounted matched sets of diluting loops (0.05 ml) and inoculating needles (20 gauge). The chain-driven carriage is programmable by use of a plastic program board read by a light-sensitive apparatus mounted on the carriage. In the course of a run, the diluting loops are filled to incandescence, cooled in sterile water, blotted dry on strips of sterile blotting paper, and lowered to pick up 0.05 ml antibiotic from discrete wells in the loading trough. Doubling dilutions are effected by lowering and rotating in wells containing sterile diluent in sequence. The wells are simultaneously filled by hydraulic pressure with 0.05 ml diluent, one row in advance of the diluting loops. Upon completion of a run, the carriage automatically returns to repeat the program. Manual control, however, is possible at any stage of the run.

II. Supplies

Clear, flat-bottom, sterile Autotrays (Cassico, Inc.)
containing 8 horizontal rows (A thru H), 15 wells each, were used for antibiotic dilutions. Following preparation, the plates were immediately sealed with tape (Candaes, Inc.) and replaced in plastic bags. These were kept at -20°C for a period not exceeding 2 weeks. Once thawed, any unused plates were discarded.

III. Antibiotics

Penicillin G (Wyeth)
Streptomycin (Pfizer)
Gentamicin (Serening)
Vancomycin (Lilly)
Ankacin (Bristol)
Sisomycin (Serening)

All antibiotics were weighed on a Mettler balance with corrections made according to stated activities. The aminoglycosides were diluted in 0.1M phosphate buffer (pH 7.4), and vancomycin and penicillin were diluted in triple distilled water. Final stock concentrations were 4,096 μg/ml for the aminoglycosides and 1,280 μg/ml for penicillin and vancomycin. All antibiotics were sterilized by passing through a millipore (0.45 µ) filter. Stocks were stored in sterile 3 ml screw-cap vials at -20°C. All were kept frozen until needed. The penicillin stock was replaced at 2-week intervals. Any unused portions following thawing were discarded.
IV. Organisms

The streptococci used in this study were isolates from a variety of human sources by the Clinical Microbiology Laboratory, University Hospitals. They had been identified as group B streptococci by positive reactions on bile esculin agar and modified salt broth. All isolates were biochemically sub-specified and maintained on 5% sheep blood agar at 4°C, with periodic subculturing to confirm purity. Reference strains were obtained from the American Type Culture Collection (ATCC, Rockville, Md.), and included Streptococcus faecalis (ATCC #19433), S. faecalis var. zymogenes (ATCC #10100), and S. faecium (ATCC #19434).

V. Miscellaneous Materials

A. Multipronged wire inoculator (Camlab, Inc.)
B. 150 mm sheep blood (5%) agar plates (Gibco)
C. 0.05 ml calibrated reusable dropers (Scientific Products)
D. Autotray viewer (Camlab, Inc.)
E. Compressed air source
F. Autotray sealing tape (Camlab, Inc.)
G. Sterility hood.
METHODS

I. Subspeciation

All clinical isolates were subspecified on the basis of a minimum number of biochemical tests (Table 1) suggested by Facklam et al (11, 12). All media were sterilized for 15 minutes at 15 psi (121°C) unless otherwise noted, and the pH determined by a Corning expanded scale pH meter. The media were inoculated with two drops of an overnight culture in brain-heart infusion (BHI, Gibco) broth using sterile Pasteur pipettes, and incubated at 35°C.

Bile esculin agar (11) was dispensed in 100 mL petri plates, and, following inoculation, the plates were read at 24 and 48 hours. Blemshening of the agar was considered a positive reaction.

The modified salt broth (11) was prepared by adding 60g NaCl, 1g dextrose, and 25 mL heart infusion broth (HIB, Gibco) to 1 liter of water. One mL indicator solution (1.6g bromcresol purple in 100 mL 95% ethanol) was added prior to autoclaving. The medium was dispensed in 3 mL aliquots. Growth accompanied by a moderate color change (yellow-brown) after 48 hr. incubation was considered to be a positive reaction.

Gelatin medium was prepared by adding 120g gelatin (Gibco) to 1 liter HIB, and dispensed in 5 mL aliquots into screw-cap tubes. Hydrolysis was determined following 72 hr. incubation by placing the gelatin tests and an uninoculated
### TABLE 1. Biochemical differentiation of the group D streptococci.

<table>
<thead>
<tr>
<th>Test</th>
<th>S. faecalis</th>
<th>S. faecalis var. arabinosus</th>
<th>S. intermedius</th>
<th>S. constellatus</th>
<th>S. dysgalactiae not enterocolitici</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,14-esculin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Modified salt broth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B hemolysis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from: mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>glycerol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>arabinose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>sucrose</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
</tbody>
</table>

*a Symbols: +, positive reaction; -, negative reaction; V, variable reaction

*b Horse blood agar plates
control tube at 4°C. Following solidification of the control tube, tubes which remained liquid were recorded as positive for gelatin hydrolysis.

Beta hemolysis was determined by streaking and cutting into prepared 5% horse blood agar (Gibco) plates.

The sugar broths were made in 1% concentrations, 100 ml of a 10% solution of each sugar being added to 900 ml BHI.

One ml indicator solution (above) was added, and the media autoclaved for 10 minutes at 15 psi in 3 ml aliquots. Tests were read at 24, 48, and 72 hours, and positive reactions were recorded for those showing a change in the indicator from purple to yellow due to acid production.

II. MIC-MIC Determinations

Stock concentrations of the aminoglycosides (4,096 µg/ml) and vancomycin and penicillin (1,280 µg/ml) were thawed and diluted 1:2 in sterile BHI broth. Following sterilization, the Autotiter's injector system was mounted on the machine and primed with sterile distilled water. The injector needles were checked for retention of vacuum, and then calibrated to deliver 0.05 ml each by adjustment of the injector barrel stroke. The system was then flushed and filled with sterile BHI broth, and the diluting loops sterilized by flaming to incandescence. The program board (Run #1)
was keyed to dilute the antibiotics in doubling dilutions from row 1 through row 15 in all horizontal rows (A-H).

Since there is a method-dependent 1:4 dilution of the antibiotic between the loading through and Autotray, final concentrations ranged from 512 to 0.06 µg/ml for the aminoglycosides, and from 160 to 0.03 µg/ml for vancomycin and penicillin. Row 15 contained only BH1 broth, the upper 2 wells being growth controls; the remaining 6 being sterility controls.

Following preparation, the plates were immediately sealed and stored at -20°C for a period no longer than 2 weeks.

Lactobacilli, following overnight incubation in 3 ml BH1 at 35°C, were diluted 1:1000 in sterile BH1 to yield an inoculum of approximately 10^5 CFU/ml. Using calibrated 0.05 ml droppers, one drop per well was delivered into a plate which had been allowed to thaw at room temperature. Incubated plates were resealed and incubated 18-24 hr. at 35°C. They were then read on the Autotray viewer (Fig. 3), the well in each row having the lowest concentration which still inhibited growth (read as turbidity or more than 3 wells of growth) being considered the MIC for that strain.

MIC's were determined by use of the multipronged wire inoculator, which was planted to incendence (Fig. 4), air-cooled, and placed onto the Autotray. Subsequent transfer was to a 150 mm sheep blood agar plate, where the inocula were
Fig. 1. The Abney viewer, showing reading of representative W.50 values.
deposited (Fig. 5) by gently pressing and spreading with a slight motion of the wrist. Following incubation at 35°C for 18-24 hr., the MBC was determined to be the lowest concentration which showed complete lack of growth on the agar plate (Fig. 6).
III. Synergism Determinations (Micromethod)

The addition of 2 antibiotics to each well of the autotray required separate runs on the Autotitter. The first antibiotic was added to all 8 wells of the loading trough in the same concentration (256 µg/ml), and was serially diluted through row 1/4 by use of the program board as described above (Run 1). Following the first run, plates were stacked inside a sterility hood (White-Roseette, Ultrasonic Industries, Inc.) until all had been completed.

The addition of the aminoglycoside required doubling dilutions to be made from 256 µg/ml to 2 µg/ml. These were then pipetted into the wells of a new loading trough in descending order and, following sterilization of the diluting loops to remove all traces of the former antibiotic, were each serially diluted through well 1/4 in their respective rows.

The dilution of this second set of antibiotics required no additional diluent (total volume was maintained at 0.05 ml/well), and was controlled by a second program board (Run 2). This left a pattern approximating that of the checkerboard configuration, with vancomycin/aminoglycoside concentrations ranging from 256/128 µg/ml to 0.0001 µg/ml (Fig. 7). Following this run, the plates were sealed and stored within plastic bags at -20°C for no longer than 2 weeks.
Inoculation of the plates was identical to the procedure (above) for the MIC determinations, as was inoculation of the large blood agar plates with the multipronged wire inoculator for bactericidal determinations.

IV. Synergism Determinations (Macromethod)

Serial doubling dilutions ranging from 256 to 8 μg/ml were made of penicillin and streptomycin in BHI. Forty-eight sterile tubes were arranged in a square, the vertical rows receiving identical, decreasing concentrations of streptomycin in 0.5 ml aliquots; and the horizontal rows, decreasing concentrations of penicillin in 0.5 ml aliquots. One ml of a 10⁻³ dilution of overnight culture was added to all tubes, the final dilutions resulting in a checkerboard configuration. Following incubation for 18-24 hr at 35°C, tubes showing growth had 0.01 ml subcultured to blood agar plates and reincubated another 18 hr. The MBC was considered the combination of lowest concentrations showing total inhibition of growth.

V. Inoculum Size

Samples were randomly picked weekly to be checked for inoculum size. One drop from the calibrated dropper (0.05 ml) of the 1:1000 dilution of overnight culture was added to 9 ml
sterile unsolidified HI agar, thoroughly mixed, and
poured. Following incubation at 35°C for 18-24 hr, total
colonies were counted and calculations made to determine
CFU/ml.

VI. Dilution Accuracy

The tray well dilutions were assayed for reproducibility
and accuracy by means of an agar diffusion test using
Bacillus-seeded agar. Wells were punched into seeded plates and
filled with 0.01 ml sample from the Autotray. A series of
wells containing known concentrations of gentamicin were
assayed simultaneously on the same plate, and the 2 sets of data
then plotted on semi-log paper for comparison (Fig. 8).
Fig. 9. Comparison of dilution accuracy between standard tube method (Macromethod) and Autokiter (Micromethod) using the seeded-spor technique.
RESULTS

Of the 86 strains used in this study, 41 (47%) were determined to be *S. faecalis* var. *liquefaciens*: 21 (24%), *S. faecalis*: 16 (19%), *S. faecalis* var. *zygosporus*: 2 (2%) *S. faecium*; and 6 (7%), group D streptococci, not enterococci. Fifty-three isolates were obtained from urine specimens, 15 from wounds, 6 each from blood and sputum, and 3 were reference strains obtained from the ATCC (Table 2).

Aminoglycoside MIC data (Fig. 9) show sisomicin to be most active, inhibiting 76 (88%) of the 86 strains at a concentration of 64 µg/ml. Gentamicin is nearly as effective, inhibiting 63 (73%) at the same concentration. Amikacin inhibits only 20 (23%) strains, and both streptomycin and tobramycin inhibit only 2 (3%) strains at this concentration. At a level of 512 µg/ml, gentamicin and sisomicin inhibit all 86 (100%) strains, and amikacin inhibits 84 (96%). Streptomycin inhibits 60 (69%) and tobramycin 51 (59%).

The MIC values for the aminoglycoside antibiotics (Fig. 9) show an average 2 to 4-fold dilution increase over the MIC values. The order of effectiveness remains the same, sisomicin and gentamicin killing 71 (83%) and 50 (58%) strains, respectively, at a concentration of 64 µg/ml. At this same concentration, amikacin is bactericidal for 13 (15%) strains, and tobramycin and
TABLE 2. Sources of 86 group D streptococci according to species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wound</td>
<td>sputum</td>
<td>blood/Fluid</td>
<td>urine</td>
<td>reference³</td>
</tr>
<tr>
<td>S. faecalis</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1c</td>
<td>1</td>
</tr>
<tr>
<td>S. faecalis var. liquidus</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>S. faecalis var. pyrogenes</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>S. faecium</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Group D, not enterococcus</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

³ Reference strains obtained from ATCC.
streptomycin for 8 (2%) strains. At a concentration of 512 µg/ml, aminopenicillin and gentamicin are bactericidal for all 86 (100%) strains, and amikacin for 84 (98%). Streptomycin at the same concentration kills 60 (70%) of tetracycline, 36 (42%) of these, the 84 strains, respectively, at a concentration of 0.5 µg/ml. However, neither antibiotic is consistently bactericidal at lower concentrations. At a concentration of 40 µg/ml, penicillin kills 61 (72%) strains; and vancomycin, 71 (81%). At a concentration of 160 µg/ml, both antibiotics are similarly effective, penicillin killing 69 (80%) and vancomycin, 66 (78%) of the total strains.

Correlation between the standard tube dilution and semi-automated techniques for synergy determinations were evaluated by a series of three repetitions of each over a two-month period. Four enterococci were selected to be simultaneously tested by each method for synergy to a combination of penicillin and streptomycin. Two strains were highly resistant and non-synergistic (by standard technique), and the remaining two were synergistic at relatively low concentrations of each antibiotic (Table 3). Accuracy of the microdilutors on the Autotiter III for delivery of stated volumes was verified by microbiological assay, transferring contents of autotiter wells to wells cut in agar plates seeded with a sensitive Bacillus cereus. Standard concentrations were simultaneously run
<table>
<thead>
<tr>
<th>Method</th>
<th>Strain 1410</th>
<th>Strain 5247</th>
<th>Strain 100</th>
<th>Strain 19433</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
</tr>
<tr>
<td>Autotray (using multi-prong wire inoculator)</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
</tr>
<tr>
<td></td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
</tr>
<tr>
<td>Autotray (using Pasteur pipetting to inoculate)</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
</tr>
<tr>
<td></td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
<td>32/64</td>
</tr>
<tr>
<td>Standard Tube Dilution</td>
<td>32/32</td>
<td>32/32</td>
<td>32/32</td>
<td>32/32</td>
</tr>
</tbody>
</table>

* Repeated at 4-week intervals

** MEC expressed in μg/ml penicillin (upper number) and streptomycin (lower number)
and curves plotted on semi-log paper showed less than a 1/4 deviation between standards and test. Fresh antibiotic stocks were prepared prior to each set of tests. In addition to obtaining bactericidal results from the Autotry with the multi-prong wire inoculator, samples were also planted singly from each well using sterile Pasteur pipets to deliver approximately 0.1 ml. Both strains 1410 and 5347 were found to be non-synergistic, and resistant to concentrations greater than 30 µg/ml for each antibiotic by all 3 methods of testing for all 3 repetitions. Strain 100, in all 3 sets of tests and by all 3 methods, was susceptible to a concentration of 10 µg/ml penicillin. The streptomycin concentration varied from 0.25 µg/ml to 4.0 µg/ml between both sets of tests and methods of testing. Strain 19433 likewise showed a consistent susceptibility to penicillin, the concentration being 6.0 µg/ml for both Autotry methods and 10 µg/ml by the standard technique. Again streptomycin concentration was variable, ranging from 1 µg/ml to 4.0 µg/ml with no pattern between sets of tests and test methods used.

The isolates were divided according to species to determine if there were any species-determined patterns of resistance. Each MBC curve was examined at its point of steepest incline, where a species-resistant trend would be most evident. Thus, end-point concentrations chosen for examination were: 160 µg/ml, vancomycin; 128 µg/ml, gentamicin, sisomicin, and amikacin; and 512 µg/ml,
TABLE 4: Comparison of the cumulative percent of enterococci killed, according to species, by different antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><strong>H. Faecalis</strong>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><strong>H. Faecalis var. intermedium</strong>&lt;sup&gt;b&lt;/sup&gt;</th>
<th><strong>H. Faecalis var. Prasitica</strong>&lt;sup&gt;b&lt;/sup&gt;</th>
<th><strong>H. Faecium</strong>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalidixic acid&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77 (27)</td>
<td>76 (1)</td>
<td>9 (1)</td>
<td>50 (1)</td>
</tr>
<tr>
<td>Streptomycin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67 (1)</td>
<td>65 (2&lt;sup&gt;7&lt;/sup&gt;)</td>
<td>62 (10)</td>
<td>100 (2)</td>
</tr>
<tr>
<td>Gentamicin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 (17)</td>
<td>90 (37)</td>
<td>88 (1&lt;sup&gt;7&lt;/sup&gt;)</td>
<td>100 (2)</td>
</tr>
<tr>
<td>Amikacin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 (12)</td>
<td>80 (2&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>80 (1&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>90 (1)</td>
</tr>
<tr>
<td>Tetracycline&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43 (9)</td>
<td>40 (1&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>50 (8)</td>
<td>100 (2)</td>
</tr>
<tr>
<td>Sisomisin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 (19)</td>
<td>100 (41)</td>
<td>88 (11)</td>
<td>100 (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of strains per species tested
<sup>b</sup> Total number of strains killed in parentheses
streptomycin and tobramycin. As seen in Table 4, the relative
susceptibilities of the various species to the different anti-
biotics are quite similar. However, the pattern of S. Pneumoniae
cannot be assessed from these data, due to the lack of statistically
significant number of strains.

The effect of penicillin in combination with streptomycin on
ererococci is shown in Figure 11. At a concentration of 1.0 µg/ml
streptomycin plus 6.0 µg/ml penicillin, all 66 strains were
inhibited; whereas, the same combination was bactericidal for
only approximately 60% of the total. Nearly percent of all strains
were killed by a concentration of 10 µg/ml penicillin plus 2.0 µg/ml
streptomycin, but the remaining 10% were resistant to concentra-
tions greater than 50 µg/ml penicillin and 64 µg/ml streptomycin.
Of the 66 strains, 21 (32%) showed synergism, and 65 (76%) did not.

The combination of vancomycin plus streptomycin (Fig. 12)
inhibited all 66 strains tested at concentrations of 1.0 µg/ml
streptomycin and 6.0 µg/ml vancomycin. However, the combination of
4.0 µg/ml streptomycin and 16 µg/ml vancomycin killed only 75% of
the total. Nearly 25% of all strains tested were resistant to a
combination of 32 µg/ml vancomycin and 16 µg/ml streptomycin. Of
the 66 strains, 51 (77%) showed synergism, and 15 (23%) did not.

Gentamicin in combination with vancomycin (Fig. 13) in con-
centrations of 1.0 µg/ml gentamicin and 6.0 µg/ml vancomycin
inhibited all 66 strains, and was also bactericidal for more than
Fig. 11. Cumulative percent of 86 strains of enterococci inhibited (A) and killed by (B) various concentrations of streptomycin in the presence of increasing concentrations of penicillin.
Fig. 12. Cumulative percent of 66 strains of enterococci inhibited by (A) and killed by (B) various concentrations of streptomycin in the presence of increasing concentrations of vancomycin.
Fig. 13. Cumulative percent of 56 strains of enterococci inhibited (A) and killed by (B) various concentrations of gentamicin in the presence of increasing concentrations of vancomycin.
90% of the total. All 86 were killed by a combination of 16.0 μg/ml vancomycin plus 1.0 μg/ml gentamycin. Additionally, of 86 strains, 74 (85%) demonstrated synergy, and 12 (15%) did not.

1.0 μg/ml amikacin combined with 6.0 μg/ml vancomycin (Fig. 14) inhibited all 86 strains tested, but was bactericidal for only 70%.

A combination of 16.0 μg/ml vancomycin plus 5.0 μg/ml amikacin killed slightly more than 90%, the remainder being resistant to concentrations in excess of 32 μg/ml vancomycin and 64 μg/ml amikacin.

Sixty-five (75%) strains demonstrated synergy; 21 (25%), did not.

Tobramycin in combination with vancomycin (Fig. 15) in a concentration of 1.0 μg/ml tobramycin plus 6.0 μg/ml vancomycin was inhibitory for all 86 strains, but the same combination was bactericidal for slightly more than 60% of the total. All strains were killed by a combination of 16.0 μg/ml vancomycin and 1.0 μg/ml tobramycin. Seventy-four (86%) strains were synergistic; 12 (14%), were not.

Sisomicin combined with vancomycin (Fig. 16) inhibited all 86 strains at a concentration of 0.5 μg/ml sisomicin plus 5.0 μg/ml vancomycin; whereas, the same amount killed less than 30% of the total. A combination of 0.0 μg/ml sisomicin and 16 μg/ml vancomycin killed approximately 50%, the remainder being resistant to concentrations greater than 32 μg/ml vancomycin and 64 μg/ml sisomicin.

Sixty-three (73%) of the strains demonstrated synergy; 23 (27%), did not.
Fig. 14. Cumulative percent of 66 strains of enterococci inhibited (A) and killed by (B) various concentrations of amikacin in the presence of increasing concentrations of vancomycin.
Figure 15. Cumulative percent of germination of uninoculated (a) and killed (b) plants in the presence of increasing concentrations of vancomycin.
Fig. 16. Cumulative percent of 86 strains of enterococci inhibited (A) and killed by (B) various concentrations of sisomicin in the presence of increasing concentrations of vancomycin.
The various combinations of antibiotics were examined to determine whether the presence or absence of high-level resistance (i.e. greater than 512 µg/ml) to the aminoglycoside part of the combination could be used as a valid marker for prediction of probably synergy (Table 5). As seen, 69% of the strains tested were of moderate level resistance to streptomycin, only 17% of these being synergistic in the combination of streptomycin with penicillin. Conversely, of the 31% possessing high-level resistance to streptomycin, 7% still demonstrated synergy in this combination. Of the 69% which were moderately resistant to streptomycin, 90% demonstrated synergy in combination with vancomycin as did 12% of the highly resistant strains. Ninety-eight percent of all strains were moderately resistant to amikacin; of these 76% demonstrated synergy, as did the 2% which were highly resistant. Streptomycin killed all 86 strains at a concentration of 512 µg/ml, but 77% failed to demonstrate synergy. Forty-six percent of all strains were moderately resistant to tobramycin; 36% demonstrated synergy. Moreover, of the 5% which were highly resistant, 50% were synergistic. Gentamicin killed all strains at a concentration of 512 µg/ml, 96% of which demonstrated synergy.
TABLE 5. Cumulative percent of enterococci demonstrating synergy with regard to presence or absence of high-level resistance to the aminoglycoside portion of the antibiotic combination.

<table>
<thead>
<tr>
<th>Penicillin + Streptomycin</th>
<th>Vancomycin in combination with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;512</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>17 (15&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>50 (63)</td>
</tr>
<tr>
<td>Synergy</td>
<td>(60)</td>
</tr>
<tr>
<td>No synergy</td>
<td>72 (45)</td>
</tr>
<tr>
<td>(26)</td>
<td>(16)</td>
</tr>
</tbody>
</table>

<sup>a</sup> High-level resistance considered to be an MEC >512 µg/mL
<sup>b</sup> Total number of strains out of 86 tested
DISCUSSION

Lack of a reliable technique simpler than the standard tube dilution method for determination of synergy between antibacterial combinations has limited the number of clinical laboratories which perform these determinations. The introduction of micro-titration techniques, formerly limited to serological applications (85, 93, 14), into the bacteriology laboratory should serve to correct this serious deficiency. In this study, a programmable serial dilutor used in development of a semi-automated procedure for determination of synergy between different antibiotic combinations. Time required for a single determination was approximately 1/10 that by the standard technique. Results were shown to be accurate within the one tube dilution range of error inherent in all such determinations, in agreement with workers who have found greater than 90% correlation between micro- and macro-methods (9, 16, 29). Additionally, it was shown that the method is reproducible over a period of months, varying again no more than 10% the results by the standard technique. Plates made on the Autolizer can be maintained a minimum of 2 weeks at -20°C with no loss of antibiotic potency, allowing advance preparation in quantity.

A specific synergy study was undertaken to demonstrate the applicability of the semi-automated approach. Susceptibility of enterococci to combinations of vancomycin and the various antibiot
glyco-side antibiotics was studied to determine if any of these combinations in vitro suggest a possible alternative to the penicillin-streptomycin regimen currently used in many cases of enterococcal endocarditis. Since this disease is known to be maintained by resistant enterococci over extensive periods of time in the subacute form, inhibitory concentrations of antibiotics are of little value in eradicating the organisms. Thus the bactericidal effectiveness of various combinations of antibiotics was of greatest interest.

The MIC's of vancomycin and the aminoglycosides, considered separately, proved to be ineffective in terms of clinically achievable serum levels of the antibiotics. Although vancomycin showed promise, inhibiting 63% of the isolates at a concentration of 2.5 µg/ml, it failed to demonstrate good bactericidal activity. At an achievable serum level of 20 µg/ml (71), less than 25% of the isolates were killed.

The enterococci were similarly resistant to all aminoglycosides tested. In addition to the currently available streptomycin and gentamicin, others tested included: amikacin (formerly BB-kΩ), a semisynthetic antibiotic derived from kanamycin (4, 5, 6); tobramycin (formerly nebramycin factor 6), a fermentation product isolated from Streptomyces tobramycin (3, 41, 47, 56); and sisomicin (formerly antibiotic 6640), isolated from Micromonospora immunensis (10). Mean MIC's obtained for gentamicin and sisomicin were 64 µg/ml, while achievable serum levels were 6 µg/ml (41) to
8 μg/ml (48, 55) for gentamicin and for amikacin (69). Similarly, the mean MBC for amikacin was 120 μg/ml, with an achievable serum level of approximately 20 μg/ml (54, 59). The mean MBC values for streptomycin and tobramycin were 512 μg/ml, far above the achievable serum levels of 40 μg/ml for streptomycin and 5 μg/ml (31, 46) to 8 μg/ml (55) for tobramycin.

These antibiotics in combination, however, produced a much different effect. The most commonly administered combination, penicillin plus streptomycin, was run to serve as a correlation between published data and the new method. By the semi-automated procedure, 24% of all enterococci tested were synergistic, in good agreement with prior reports (54). In comparison, 63% were synergistic to the combination of vancomycin plus streptomycin. Seventy-six percent of the strains tested were sensitive to the combination of vancomycin and amikacin. Sisomicin, on the basis of its low (in comparison to the other aminoglycosides) MBC values, was projected to be an excellent combination with vancomycin; however, only 73% of the strains were sensitive to this combination. On the other hand, tobramycin, on the basis of its relatively poor bactericidal properties against enterococci, was projected to be a poor combination with vancomycin. Eighty-six percent of the enterococci, however, were found to be synergistic. Similarly, 86% of all strains were also synergistic to the combination of gentamicin plus vancomycin.
Earlier reports have been contradictory (62, 70) concerning prediction of the probability of synergy on the basis of the susceptibility of the strain in question to a high dosage of the aminoglycoside half of the combination. Accordingly, every strain was examined for susceptibility to 512 μg/ml of each aminoglycoside antibiotic, and also for synergistic reaction to that antibiotic in combination with vancomycin. It was found that in all combinations at least 14%, and up to 27% of the strains were non-synergistic.

On the basis of susceptibility of the antibiotic in high concentration, the opposite would have been expected. From these results it must be concluded that although the high dosage susceptibility test may in some cases be a valid indicator, it cannot replace performance of the synergy test.

It has been suggested (11, 63) that speciation of enterococci serves no useful purpose in the clinical laboratory. In support of this, it was found that there were no significant differences among the species when compared at the concentration showing the sharpest rise in cumulative percent affected. Lower or higher points on the curve would not have reflected any variations among species susceptibilities. It would appear S. faecium may be a slightly more sensitive strain than the other enterococci, as reported in an earlier study (76); however, as mentioned, there are not enough strains in this study to be statistically valid.
CONCLUSIONS

The semi-automated micromethod proposed for determination of synergy of antibiotics in combination was as accurate and reproducible as the tube dilution checkerboard micromethod. The semi-automated micromethod had advantages over the micromethod in that it required approximately one-tenth the time to prepare and store a large number of tests in advance of need. Additionally, reagent expenditure was considerably less by the micromethod.

These advantages made the micromethod particularly well suited for the survey of groups of bacteria for their susceptibility to antibiotics used singly or in combination. Because both MIC and MBC values are required for antibiotics used singly and in combination, large numbers of individual tests must be performed. The value of the semi-automated microdilution method was demonstrated by the fact the survey of the 86 strains of enterococci for susceptibility to five aminoglycoside antibiotics in combination with vancomycin would have required approximately 72,000 tubes using the micromethod, opposed to 600 autotrays by the micromethod. Approximately 900 hours of labor would have been required against the approximately 100 hours for the micromethod.

The results of the survey of enterococci demonstrated the value of obtaining sufficient data to recognize the effectiveness of new antibiotic combinations (96% synergy vancomycin-gentamicin),
and to document the loss of effectiveness of older combinations (20% synergy penicillin-streptomycin). It was interesting to note that all the newer aminoglycoside antimicrobials tested had greater synergistic activity with vancomycin than did streptomycin (gentamicin, 96%; tobramycin, 86%; amikacin, 76%; and sisomicin, 73%).


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