
Failure to Demonstrate a Consistent In Vitro Bactericidal Effect of Trimethoprim-Sulfamethoxazole against Enterococci

AMIAD NAJJAR AND BARBARA E. MURRAY

Department of Medicine and Program in Infectious Diseases and Clinical Microbiology, University of Texas Medical School, Houston, Texas 77030

Received 28 October 1986/Accepted 6 February 1987

Controversy exists as to the in vitro and in vivo activities of trimethoprim-sulfamethoxazole (TMP-SMX) against enterococci. In this study, we investigated the in vitro activity of TMP-SMX in the type of Mueller-Hinton broth previously reported to give the lowest MICs and MBCs with enterococci. In all instances, MICs were \( \leq 0.5 \) \( \mu \)g/ml. The majority of tests showed MBCs of \( >32 \) \( \mu \)g/ml, although there was some effect from varying the inoculum and the length of incubation after subculturing. Minor differences were noted when tests were repeated and between the results from microdilution and macrodilution tests and those obtained by the time-kill method. These results, as well as other reports, suggest that TMP-SMX should not be considered a reliable bactericidal agent against enterococci.

Enterococci are intrinsically resistant to various antimicrobial agents including clindamycin, aminoglycosides, cephalosporins, and others (5, 10). Even the most active agents, such as penicillin G and ampicillin, are characteristically bacteriostatic; although these agents are adequate for soft tissue infections, optimal therapy for enterococcal endocarditis consists of a bactericidal regimen such as penicillin and an aminoglycoside (3, 11). A number of reports have now documented strains with high-level resistance to all aminoglycosides and, as a consequence, lack of a synergistic, bactericidal effect when combined with penicillin (4, 8). Plasmid-mediated beta-lactamase production has been observed in two of these strains (6, 7). For these reasons, there is renewed interest in agents active against enterococci, particularly those agents which have bactericidal activity. A recent report by Crider and Colby suggested that in certain media (Mueller-Hinton broth [MHB]; Difco Laboratories, Detroit, Mich.), trimethoprim-sulfamethoxazole (TMP-SMX) was bactericidal at low concentrations against enterococci (1). However, a clinical report of sepsis developing during TMP-SMX therapy of urinary tract infections (2) and a report showing diminished activity of TMP-SMX in urine (12) have raised questions about the clinical applicability of this observation. In this study we investigated possible bactericidal activity of TMP-SMX against enterococci in Difco MHB by using different methods and with different inocula.

Twenty strains of Streptococcus faecalis from previous studies were used (4, 8). Three strains had high-level resistance (MIC, >2,000 \( \mu \)g/ml) to streptomycin, gentamicin, and all other aminoglycosides, and 1 strain produced penicillinase (7); 10 strains were highly resistant to gentamicin but not streptomycin, 2 strains were highly resistant to streptomycin but not gentamicin, and 5 strains had no high-level aminoglycoside resistance. Since the presence of high-level aminoglycoside resistance was found to have no effect on the results obtained with TMP-SMX, results from the various groups were considered together. Many strains were tested more than once with the same or different inocula. When reporting the results, we indicated both the number of strains and the number of strain-drug interactions when the latter included repeat testing of some of these strains at different times. MHB (control 668270) was purchased from Difco. Difco MHB (control 704107) was previously shown to be superior to MHB from GIBCO Diagnostics, Madison, Wis., or from BBL Microbiology Systems, Cockeysville, Md., for demonstrating bactericidal activity against enterococci (1). This medium was supplemented with Mg\(^{2+}\) and Ca\(^{2+}\) and adjusted to pH 7.4 as described by Crider and Colby (1). TMP-SMX, a gift from Burroughs Wellcome Co., Research Triangle Park, N.C., was used in a ratio of 19:1; the concentrations given here refer to the concentration of TMP.

We first tested all strains by the microdilution method, using a final volume of 200 \( \mu \)l per well (9). The inoculum was determined by serial dilution and colony counts of each strain. Serial twofold dilutions of TMP-SMX were used in concentrations of 0.0625 to 32 \( \mu \)g of TMP per ml. MICs were read after incubation for 20 to 24 h at 37°C. MICs determined by microdilution were \( \leq 0.0625 \) \( \mu \)g of TMP per ml for all except three strains (MICs of 0.25, 0.5, and 0.5 \( \mu \)g/ml). Many strains were tested at different inocula (from 10\(^4\) to 10\(^6\) CFU/ml), and the MICs were not affected by the size of the inoculum. Three strains were also tested by macrodilution testing in a final volume of 2 ml, and MICs for these strains were \( <0.03 \) \( \mu \)g/ml.

For MBC determinations by the microdilution method and with inocula of \( \geq 10^6 \) CFU/ml, 20 \( \mu \)l was carefully removed from the center of the well without shaking the tray and was plated onto brain heart infusion agar (BHIA). For wells inoculated with \( <10^6 \) CFU/ml, 20 and 100 \( \mu \)l (one-half of the original volume) were plated for colony counts. If maximal plating efficiency is assumed and the larger volume at the lowest inoculum (2 \( \times 10^4 \) CFU/ml) is used, the cutoff for bactericidal activity (99.9% kill) is 2 CFU; this cutoff is the same as that with the smaller volume at an inoculum of 1 \( \times 10^4 \) CFU/ml. There seemed to be little or no effect of antibiotic carry-over since strains which grew on the lower concentrations also grew on the higher concentrations (32 \( \mu \)g/ml) and showed equivalent counts when 20- and 100-\( \mu \)l volumes were compared. This lack of effect is presumably due to dilution of TMP-SMX into the 35 to 40 ml of agar or

* Corresponding author.
TABLE 1. Effect of inoculum on bactericidal activity*

<table>
<thead>
<tr>
<th>Inoculum (CFU/ml)</th>
<th>No. of tests showing the following activity:</th>
<th>No. of strains (no. of tests)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bactericidal*</td>
<td>Indeterminant*</td>
</tr>
<tr>
<td>1.0 x 10^6</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>(5.5-9.9) x 10^6</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>(1.0-3.4) x 10^6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>(2.0-9.9) x 10^6</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

*Microdilution method (see text for details).

Some concentrations that were bacteriostatic were scattered among others that were bactericidal; often only a few colonies produced a change in interpretation.

When a consistent bactericidal effect was seen, it was present at all concentrations through 32 µg/ml.

to the ability of BHIA to overcome the inhibitory effect of TMP-SMX. In the macrodilution method as well, 20 µl was removed and plated onto BHIA plates. All plates were incubated at 37°C and read at 24, 48, and 72 h. We observed that up to sixfold more colonies were present when the MBC subculture plates were read at 48 rather than at 24 h, and for about 40% of the strains, this difference changed a bactericidal interpretation to a bacteriostatic one. There was little difference between counts at 48 and 72 h, and all data presented below are based on MBC subculture plates read at 48 h. In many of the experiments, colony counts were very close to the 99.9% kill level. Results were called indeterminant when sequential wells had colony counts that fluctuated between <99.9 and ≥99.9% kill; this fluctuation was often due to a difference of only a few colonies.

At a high inoculum (10^6 CFU/ml) in the microdilution method, TMP-SMX was not bactericidal against any of the 18 strains tested in 33 strain-drug interactions (MBC, >32 µg/ml) (Table 1). Of these 18 strains, 12 were also tested at an inoculum of 5.5 x 10^5 to 9.9 x 10^5, and TMP-SMX was not bactericidal at up to 32 µg/ml at this inoculum volume in any of 16 strain-drug interactions. Ten of these strains and two others were tested at an inoculum of 1.0 x 10^5 to 5.5 x 10^5. TMP-SMX was bactericidal at <0.25 µg/ml against five strains; four of these five strains were retested and gave a bacteriostatic (one strain) or indeterminant (three strains) response. Twenty-eight strain-drug interactions with 14 strains were performed with 2.0 x 10^5 to 9.9 x 10^4 CFU/ml. TMP-SMX was bactericidal at ≥0.25 µg/ml against six strains; when five of these six strains were retested, the resulting interaction was bactericidal (three strains), bacteriostatic (one strain), or indeterminant (one strain).

Time-kill curves were determined with two strains (A98 and A136) by using 20 ml of supplemented MHB in a 250-ml flask with 1 µg of TMP and 19 µg of SMX per ml. Samples (0.5 ml) were removed at 0, 4, and 24 h and serially diluted, and 25 µl was then plated onto a BHIA plate for colony counts, including, where appropriate, samples from the flask itself. The lowest detectable number of bacteria by this method is 40 CFU/ml (log_{10} 40 = 1.6), with direct sampling of the flask and maximal plating efficiency assumed; on the basis of colony counts from undiluted and diluted specimens, there appeared to be little or no antibiotic carry-over effect. The differences with high (10^7 CFU/ml) and low (10^5 CFU/ml) inocula and the occurrence of killing very close to the 99.9% level are shown in the time-kill curve in Fig. 1. Both strains showed >99.9% kill with the 10^6 CFU/ml inoculum. Strain A136 showed less than a 10-fold killing effect at 10^7 CFU/ml, whereas with A98, killing was exactly 99.9%. Although this kill rate could be defined as bactericidal, there were still 10^6 CFU/ml remaining. In microdilution tests, A136 had shown the following interactions: bacteriostatic at 10^6 CFU/ml (MBC, 32 µg/ml) and both bactericidal (MBC, ≤0.25 µg/ml) and indeterminant at 1 x 10^3 to 5.4 x 10^2 CFU/ml. With A98, there were two bacteriostatic and one indeterminant interaction at 10^6 CFU/ml, one bactericidal and one indeterminant interaction at 5.5 x 10^5 to 9.9 x 10^5 CFU/ml, and one bactericidal interaction at 1 x 10^5 CFU/ml. With the microdilution test and an inoculum of 5 x 10^5 to 7 x 10^5 CFU/ml, the combination was not bactericidal, producing 99% killing for A136 and 99.8% killing for A98 at up to 32 µg/ml. TMP-SMX was also not bactericidal by the macrodilution method against a third strain tested with this inoculum.

In conclusion, unlike previous results (1), our results in this study did not demonstrate consistent bactericidal activity of TMP-SMX against enterococci. We also showed an inoculum effect such that with ≥5.5 x 10^5 CFU/ml, no bactericidal activity was seen at any concentration tested in the microdilution system even though the optimal brand (Difco) (1) was used; at lower inocula of 2 x 10^5 to 5.4 x 10^5 CFU/ml, 14 of 26 interactions (representing eight strains) were defined as bactericidal at ≤0.25 µg/ml, particularly at the lowest inocula. Reading the subculture plates for MBC determination at 24 h would have led us to interpret 40% more strain-drug interactions as bactericidal and may explain previous reports of low MBCs (1); these authors also used BHIA and read the plates at 24 to 48 h but did not comment on differences. We also used a different lot number of MHB, which may have led to differences in bactericidal activity, since other brands of MHB do not give bactericidal activity (1). The differences among repeated tests and between the time-kill data for A98 at 10^7 CFU/ml and the other two methods at lower inocula may be due to the fact that in vitro killing often observed near the 99.9% level so that even small errors in volumes, or the presence of chaining which may make colony counts inexact, would alter the interpretation. Our results, together with those of Goodhart (2) and Zervos and Schaberg (12), suggest that TMP-SMX should not be considered a bactericidal agent against enterococci unless further testing with animal models shows its efficacy.

FIG. 1. Time-kill curves of strains A136 and A98 without antibiotics (---) and with 1 and 19 µg of TMP and SMX, respectively, per ml (-----). The lowest detectable number of bacteria was 40 CFU/ml (log_{10} 40 = 1.6).
Moreover, even excellent in vitro activity does not always correlate with in vivo activity and clinical outcome.

**LITERATURE CITED**


