

## Effect of Minimal Amounts of Thymidine on Activity of Trimethoprim-Sulfamethoxazole against *Staphylococcus epidermidis*

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**The antibacterial activity of trimethoprim-sulfamethoxazole against 99 strains of *Staphylococcus epidermidis* was tested on media known to be low in thymidine content, as determined by screening with *Streptococcus faecalis*. Eighty-one percent of the isolates were susceptible by agar dilution. Trimethoprim-sulfamethoxazole was bactericidal against two strains of *S. epidermidis* when thymidine phosphorylase was added to the medium, indicating utilization of minimal amounts of thymidine that were undetected by screening. Because bacteria vary in their utilization of thymidine and body tissues vary in thymidine content, in vitro susceptibility tests may not correlate with in vivo bactericidal activity.**

Trimethoprim-sulfamethoxazole (TMP-SMX) is effective against a wide range of bacteria. It inhibits the synthesis of tetrahydrofolate, a cofactor for the synthesis of thymidine. Recently, it has been considered as an alternative drug for the treatment of serious staphylococcal infections, including meningitis (8; H. C. Neu, *Antimicrob. Newsl.* 1:47-48, 1984). There is limited information on the in vitro activity of TMP-SMX against *Staphylococcus epidermidis*. In this study we examined the inhibitory and bactericidal activities of TMP-SMX on clinically significant isolates of *S. epidermidis*.

### MATERIALS AND METHODS

**Bacterial strains.** The 99 strains of *S. epidermidis* studied were single isolates from peritoneal dialysates from patients with peritonitis submitted to the Microbiology Laboratory, St. Joseph's Hospital, Hamilton, Ontario, Canada. They were identified by using the simplified scheme described by Kloos and Schleifer (6). *Streptococcus faecalis* ATCC 29212 was also tested. All strains were maintained at -50°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) plus 15% glycerol until tested.

**Antimicrobial agents.** TMP was supplied by Burroughs Wellcome, Inc., La Salle, Quebec, Canada, and SMX was provided by Novopharm Ltd., Scarborough, Ontario, Canada. Each drug was of known potency and freshly prepared in the appropriate diluent (9) before use.

**Antimicrobial susceptibility testing.** Agar dilution MICs of TMP, SMX, and TMP-SMX (1:19) against *S. epidermidis* strains were performed by the method described by the National Committee for Clinical Laboratory Standards (9) with Mueller-Hinton agar (MHA; lot E6DNJV; BBL) supplemented with 5% lysed horse blood (LHB) (Qualicum, Ottawa, Ontario, Canada). The plate media were stored at 5°C and used within 24 h. The MIC was taken as the lowest concentration of drug that completely inhibited growth or caused a sudden sharp (80 to 90%) diminution of growth (9).

Broth dilution MICs and MBCs were determined by the method of Schoenknecht et al. (12) with cation-supplemented Mueller-Hinton broth (MHB; lot 728447; Difco Labora-

tories, Detroit, Mich.) or MHB supplemented with 0.1 U of thymidine phosphorylase (TP; lot 54F-0349; Sigma Chemical Co., St. Louis, Mo.) per ml. The cation supplement contained 25 mg of Mg<sup>2+</sup> and 50 mg of Ca<sup>2+</sup> per liter. TP was added just before use. The final bacterial suspension was approximately 2.5 × 10<sup>5</sup> CFU/ml. Technical factor variations were minimized by using borosilicate glass test tubes to minimize bacterial adherence to the test tube walls, by adding the bacterial suspension below the meniscus to avoid tube wall contact, and by rinsing the pipette tip five times. The MIC was read at 20 h, and the endpoint was taken as the lowest concentration of the drug that showed no growth by inspection with the naked eye. For the determination of bactericidal activity, tubes without visible growth were vortexed for 15 s and reincubated for an additional 4 h. The number of surviving bacteria was determined by viable counts on Trypticase soy agar plates. Carry-over effects were minimized by plating five 10-fold serial dilutions of 0.100 ml from each tube so that plates growing 30 to 300 CFU could be counted. The plates were incubated overnight at 35°C, and the colonies were counted.

The kill curves were performed by the method of Schoenknecht et al. (12), with the modification that an overnight culture was used to prepare the inoculum. The turbidity was adjusted to a no. 0.5 McFarland standard, and 0.100 ml was inoculated into 10.0 ml of MHB in each tube with a precision pipette, so that the final bacterial suspension was approximately 5 × 10<sup>5</sup> CFU/ml. Replicate tube cultures were set up, one for each sampling time of 0, 2, 6, and 24 h. Each tube was inoculated with a separate pipette tip, which was washed 5 times in the broth, and contact between the tip and the tube was avoided. The tubes were incubated at 35°C. At the sampling times, the number of surviving bacteria in each tube was determined as described above.

### RESULTS

No carry-over effects were observed. The method of enumerating the surviving bacteria had the advantages that the equivalent of 0.010 ml of the original suspension from each tube was plated, as recommended by Pearson et al. (11), and that the TMP-SMX was diluted out.

*S. faecalis*. *S. faecalis* ATCC 29212 was used to determine the suitability of all the media for TMP-SMX susceptibility

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TABLE 1. TMP-SMX MICs for *S. faecalis* ATCC 29212 and two strains of *S. epidermidis*

Method	MIC ( $\mu\text{g/ml}$ ) of TMP-SMX <sup>a</sup> for:		
	<i>S. faecalis</i> ATCC 29212 <sup>b</sup>	<i>S. epidermidis</i> D112 <sup>c</sup>	<i>S. epidermidis</i> D213 <sup>c</sup>
Agar dilution (MHA-5% LHB) <sup>d</sup>	0.0312-0.59	0.0312-0.59	0.0312-0.59
Broth dilution (MHB) <sup>e</sup>	0.0156-0.30	0.0625-1.19	0.0625-1.19
Broth dilution (MHB-TP) <sup>e</sup>	0.0156-0.30	0.0312-0.59	0.0312-0.59

<sup>a</sup> TMP-SMX, 1:19 ratio.

<sup>b</sup> Recommended strain to screen media for low thymidine content (9).

<sup>c</sup> Test strains.

<sup>d</sup> MHA (lot E6DNJV; BBL) with 5% LHB.

<sup>e</sup> Cation-supplemented (25 mg of Mg<sup>2+</sup> and 50 mg of Ca<sup>2+</sup> per liter) MHB (lot 728447; Difco) alone and with 0.1 U of TP per ml.

testing. The diameters of the inhibition zones around a TMP-SMX disk (1.25 and 23.75  $\mu\text{g}$ , respectively) were 25.1 and 27.7 mm on MHA and MHA with 5% LHB, respectively. The MIC results are shown in Table 1. MICs were similar by the agar dilution and broth dilution methods. There was no difference in MICs when TP was added to MHB. They were within the range that is considered to indicate a low thymidine content of the medium (9). In the bactericidal test (Fig. 1) there was a 99% reduction of the original inoculum. The numbers of surviving bacteria did not change with increasing concentrations of TMP-SMX. *S. faecalis* grew equally well in the MHB growth control with

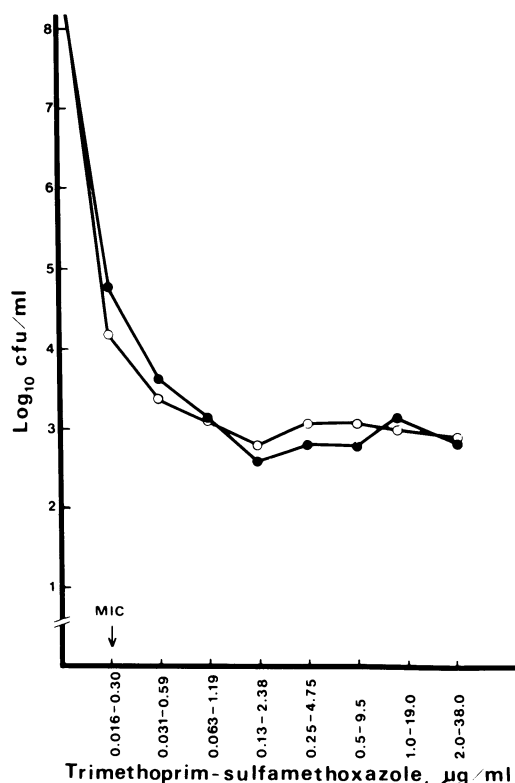


FIG. 1. Activity of TMP-SMX, at a 1:19 ratio, against *S. faecalis* ATCC 29212 in MHB without (○) and with (●) 0.1 U of TP per ml. Surviving bacteria were enumerated after 24 h of incubation. The final bacterial suspension initially contained  $4.0 \times 10^8$  CFU/ml.

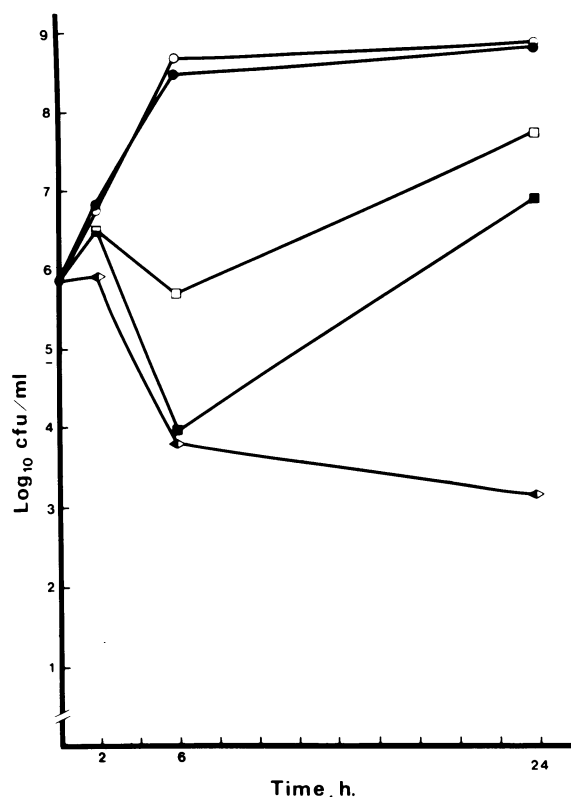


FIG. 2. Rate of killing of *S. faecalis* ATCC 29212 in MHB by TMP-SMX without and with 0.1 U of TP per ml. TMP-SMX was used at concentrations of 1.0 and 19.0 and 0.1 and 1.9  $\mu\text{g/ml}$ , respectively, without TP (▷) and with TP (◐) and 0.01 and 0.19  $\mu\text{g/ml}$ , respectively, without TP (□) and with TP (■). MHB growth controls without TP (○) and with TP (●) are also shown.

or without TP. The rate of killing of *S. faecalis* by TMP-SMX is shown in Fig. 2. At the sub-MICs of 0.01 and 0.19  $\mu\text{g/ml}$ , respectively, inhibition of growth was demonstrated initially at 6 h in MHB with TP, but this effect was reversed by the growth of *S. faecalis* to the original inoculum size by 24 h. No reversal of antibacterial activity was present at higher concentrations of TMP-SMX in MHB with TP.

*S. epidermidis*. The MICs of TMP, SMX, and the combination TMP-SMX (1:19), as determined by the agar dilution technique, against 99 strains of *S. epidermidis* are summarized in Table 2. There was a bimodal distribution of TMP

TABLE 2. Agar dilution MICs for 99 strains of *S. epidermidis* tested with TMP and the corresponding MICs of SMX and a 1:19 ratio of TMP-SMX<sup>a</sup>

No. of strains	MIC ( $\mu\text{g/ml}$ )		
	TMP	SMX	TMP-SMX
2	0.0312	64	0.016-0.297-0.031-0.59
12	0.0625	4-512	0.016-0.297-0.0625-1.19
28	0.125	4-512	0.031-0.59-0.125-2.38
30	0.25	8-512	0.031-0.59-0.125-2.38
1	0.50	16	0.031-0.59
1	8.0	128	2-38
5	32.0	512	4-76
4	64.0	32-512	1-19-4-76
16	>64.0	128->1,024	2-38-8-152

<sup>a</sup> MHA with 5% LHB was used.

MICs (range, 0.0312 to  $\geq 64$   $\mu\text{g/ml}$ ), with 74% of strains being susceptible to  $\leq 0.5$   $\mu\text{g}$  of TMP per ml and the remainder being resistant at  $\geq 8$   $\mu\text{g/ml}$ . A total of 66% of strains were susceptible to  $\leq 128$   $\mu\text{g}$  of SMX per ml, and 81% of strains were susceptible to TMP-SMX at  $\leq 2$  and 38  $\mu\text{g/ml}$ , respectively. The degree of synergy was most pronounced against those strains with comparatively high TMP MICs.

The activity of TMP-SMX against two strains of *S. epidermidis*, as determined by the tube dilution method, is shown in Table 1. The MICs in MHB with and without TP were comparable. The results of the test of bactericidal activity for *S. epidermidis* D112 are shown in Fig. 3. Similar results were seen with *S. epidermidis* D213. In MHB without TP, both *S. epidermidis* strains survived increasing concentrations of TMP-SMX, up to at least 32-fold above the MIC. The viable counts did not change. In MHB with TP there was a  $\geq 99.9\%$  reduction in the initial inoculum size by 0.13 and 2.38  $\mu\text{g}$  of TMP-SMX per ml, respectively, a twofold concentration above the MIC. *S. epidermidis* grew equally well in the MHB growth control with or without TP. The rate of killing of *S. epidermidis* D112 (Fig. 4) confirms the bacteriostatic activity of TMP-SMX in MHB without TP. MHB with TP showed bactericidal activity at 24 h but not at 6 h.

### DISCUSSION

Results of this study show that *S. epidermidis* strains evade the bactericidal activity of TMP-SMX in the presence of low levels of thymidine and that these levels cannot be detected in media by the screening method with *S. faecalis* ATCC 29212 (9).

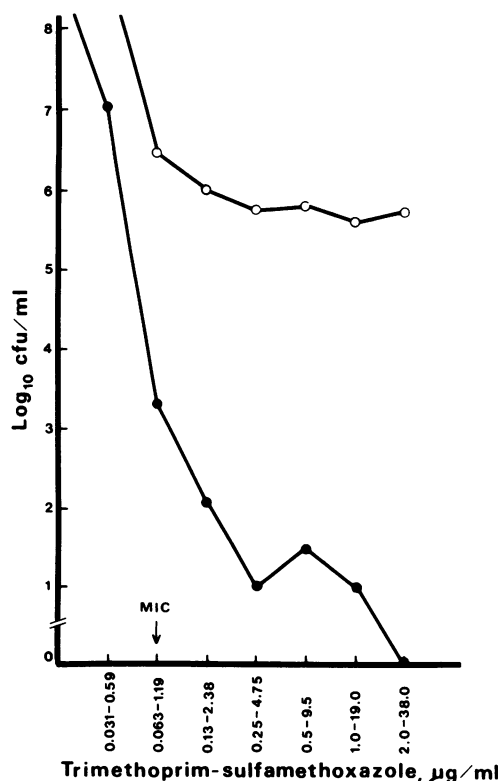


FIG. 3. Activity of TMP-SMX at a 1:19 ratio against *S. epidermidis* D112 in MHB without (○) and with (●) 0.1 U of TP per ml. Surviving bacteria were enumerated after 24 h of incubation. The final bacterial suspension initially contained  $1.8 \times 10^5$  CFU/ml.

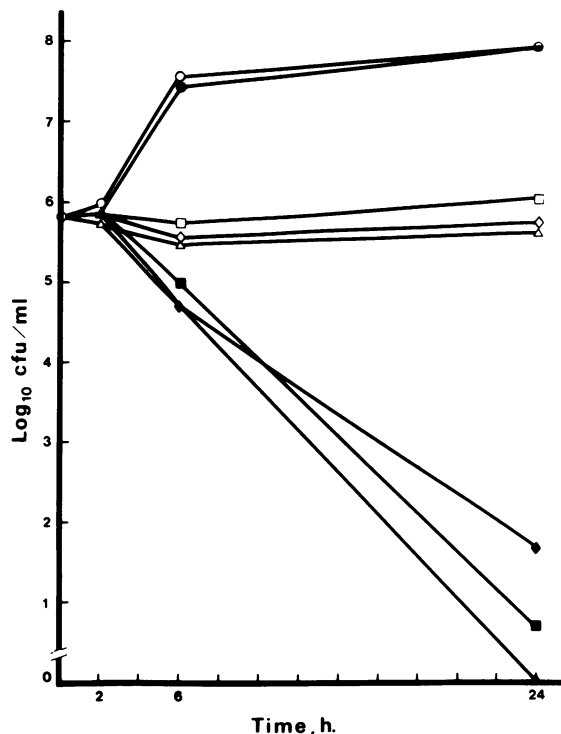


FIG. 4. Rate of killing of *S. epidermidis* D112 in MHB by a 1:19 ratio of TMP-SMX without and with 0.1 U of TP per ml. TMP-SMX was used at concentrations of 0.125 and 2.38  $\mu\text{g/ml}$ , respectively, without TP (□) and with TP (■), 0.25 and 4.75  $\mu\text{g/ml}$  without TP (◇) and with TP (◆), and 0.5 and 9.5  $\mu\text{g/ml}$  without TP (△) and with TP (▲). MHB growth controls without TP (○) and with TP (●) are also shown. The final bacterial suspension initially contained  $6.6 \times 10^5$  CFU/ml.

It is well recognized that media for susceptibility testing of bacteria to TMP-SMX should be low in thymidine content to avoid inhibition of antibacterial activity. When added to media TP or 5% LHB, a source of TP, converts thymidine to thymine and renders the media suitable for routine susceptibility tests of most bacteria except *S. faecalis*, which can utilize thymine (5). The performance of control strains of *S. faecalis* is used to assess the suitability of media for testing (9).

Bacteria vary in their ability to utilize exogenous thymidine. Some strains of the viridans group streptococci and *Streptococcus pneumoniae* cannot utilize exogenous thymidine, and thus, susceptibility testing of these organisms to TMP-SMX is not affected by thymidine content. Other organisms, such as *Escherichia coli*, *Streptococcus agalactiae*, and the enterococci, utilize various amounts of exogenous thymidine (3). The bactericidal effect of TMP-SMX against these organisms correlates directly with the concentrations of thymidine in the medium (7). Therefore, the thymidine content of media must be controlled to have standardized in vitro bactericidal tests of TMP-SMX.

Media deemed to be satisfactory by *S. faecalis* screening give reproducible results in tests of inhibition, as shown in this study. MICs for *S. faecalis* were very low, indicating thymidine levels of  $\leq 0.05$   $\mu\text{g/ml}$  in the media used in this study (2, 14, 15). MICs for *S. epidermidis* and *S. faecalis* were similar when determined by agar dilution in MHA and by broth dilution in MHB with and without TP.

Tests of bactericidal activity, however, showed that

screened MHB contained a low concentration of thymidine which enabled *S. epidermidis* strains to evade the bactericidal action of TMP-SMX. In control MHB, which was rendered thymidine free by TP, *S. epidermidis* grew very well. When TMP-SMX was added to this medium, *S. epidermidis* was killed, whereas in MHB without TP, the action was bacteriostatic. This indicates that *S. epidermidis* can utilize minimal amounts of exogenous thymidine when the synthesis of folate is blocked by TMP-SMX. *S. epidermidis* does not appear to utilize thymine produced by the action of TP on thymidine. The survival of *S. faecalis* in MHB-TP indicated utilization of thymine produced from low concentrations of the thymidine that was present (14).

The importance of thymidine-free media for bactericidal testing of TMP-SMX is clearly shown in these experiments. TP is inexpensive and can be used to make media thymidine free. Control strains of *S. epidermidis* are better indicators of thymidine content than strains of *S. faecalis* (4) because TMP-SMX activity against *S. epidermidis* is unaffected by the presence of thymine.

Bactericidal antimicrobial agents are generally advised for infections of the endocardium and bone and in immunosuppressed hosts (16). How comparable are susceptibility findings from a thymidine-free environment to those obtained under conditions that exist *in vivo*? It has been shown that TMP-SMX is rapidly lethal to a suspension of *E. coli* in sterile urine and blood obtained from healthy persons (15). The concentrations of thymidine in serum and urine from healthy persons has been shown to be  $\leq 0.02 \mu\text{g/ml}$  (10), which is too low to interfere with bactericidal activity (15). However, there are reports of the presence of thymidine in pus (1), and results of bactericidal activity tested on thymidine-free media may overestimate the susceptibility of *S. epidermidis* and other bacteria (13). They may not be clinically relevant when the treatment of serious pyogenic infections with TMP-SMX is being considered.

#### ACKNOWLEDGMENT

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