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Trimethoprim Activity in Media Selective for *Campylobacter jejuni*

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The activity of trimethoprim (TMP) in two selective media used for isolation of *Campylobacter jejuni* was evaluated. The two selective media, Campy-BAP and Skirrow medium, contain TMP in addition to other antimicrobial agents. The minimal inhibitory concentrations of TMP in blood agar base (basal agar for Skirrow medium) or brucella agar (basal agar for Campy-BAP) for three sensitive control organisms were compared with those in Mueller-Hinton agar, which contains low levels of thymidine. TMP was inactive in both blood agar base and brucella agar, even when lysed horse blood or thymidine phosphorylase was added. TMP had activity when used in combination with the other antimicrobial agents normally included in Skirrow medium and Campy-BAP, probably indicating synergism between TMP and one or more of the other antimicrobial agents. Sheep blood could be substituted for lysed horse blood in Skirrow medium without compromising the activity of TMP.

Skirrow medium (11) and Campy-BAP (1) are two selective media commonly used for isolation of *Campylobacter jejuni*. Both of these media contain trimethoprim (TMP) in addition to other antimicrobial agents. TMP is a potent inhibitor of the bacterial enzyme dihydrofolate reductase, which is responsible for the conversion of dihydrofolate to tetrahydrofolate (4, 7). This interference results in a decrease in the supply of tetrahydrofolate which has multiple effects on the bacterial cell metabolism. The reaction most severely affected is thymine synthesis (2, 8), which ultimately affects DNA synthesis. The inhibition of thymine synthesis by TMP in vitro is bypassed when extracellular thymidine is available in the culture medium (5, 8). The addition of lysed horse blood to the culture medium will remove thymidine since equine red blood cells contain thymidine phosphorylase, an enzyme which converts thymidine to thymine (5). Thymine is 100 times less active than thymidine is in interfering with the action of TMP (3, 9).

The basal media comprising Skirrow medium and Campy-BAP, blood agar base and brucella agar, respectively, contain nutritionally rich ingredients and are therefore likely to contain sufficiently high levels of thymidine to reverse the inhibitory effect of TMP. Skirrow added 7% lysed horse blood to his medium to remove thymidine, but Campy-BAP contains 10% sheep blood, which is low in thymidine phosphorylase (5). The purpose of this study was to evaluate the activity of TMP in these two nutritionally rich basal media in the presence and absence of lysed horse blood or purified thymidine phosphorylase to determine whether TMP is an effective selective agent in isolation media for *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains. Four *C. jejuni* isolates were used in this study. Of the four strains, three (strains A1446, A1256, and E6484) had been isolated at the Centers for Disease Control from patients with diarrhea, and one was a reference strain, NCTC 11168, obtained from M. B. Skirrow of Worcester, United Kingdom. In addition to the *C. jejuni* strains, four control strains sensitive to TMP were used: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, and *Proteus mirabilis* 2002-79 (Enteric Reference Laboratories, Centers for Disease Control). All cultures were stored frozen at −70°C.

Media. Three basal media were used for minimal inhibitory concentration (MIC) determinations: brucella agar (BBL Microbiology Systems, Cockeysville, Md.), blood agar base no. 2 (Oxoid Ltd., England), and Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.). One lot of Mueller-Hinton medium was used because it had proved to be suitable for TMP and sulfonamide testing in the past. The respective manufacturers were unable to provide data on the approximate thymidine content of these media, and no references were found in the literature. For MIC determinations, additives to the three basal media included lysed horse blood, sheep blood, thymidine (Sigma Chemical Co., St. Louis, Mo.), and thymidine phos-

phorylase (Burroughs Wellcome Co., Research Triangle Park, N.C.).

The Campy-BAP selective medium in this study consisted of brucella agar with 10% sheep blood and five antimicrobial agents (per liter): TMP, 5 mg; vancomycin, 10 mg; polymyxin B, 2,500 IU; amphotericin B, 2 mg; and cephalothin, 15 mg. Skirrow medium consisted of blood agar base no. 2 with 7% lysed horse blood and three antimicrobial agents (per liter): TMP, 5 mg; vancomycin, 10 mg; and polymyxin B, 2,500 IU.

Trimethoprim MICs. A standard solution of TMP (Burroughs Wellcome) was prepared in 0.1 N HCl and subjected to twofold serial dilution in 0.05 N HCl before being incorporated into the various agar media tested. The pH of the media was not affected significantly by the addition of the TMP solution.

Inocula for the control organisms were prepared by the following procedure for antibiotic sensitivity testing, which is not identical to the method used for reference MIC determinations. Five colonies were picked from an overnight plate culture of each organism (*E. coli, Staphylococcus aureus, Streptococcus faecalis*, and *P. mirabilis*) and inoculated into Mueller-Hinton broth. These broth cultures were incubated for 2 to 4 h until they showed visible growth. The broth cultures were diluted to obtain a concentration of approximately 10^7 organisms per ml by comparison with a visual turbidity standard.

Plate cultures of *C. jejuni* that had been incubated for 18 to 24 h were inoculated into brucella broth (Difco Laboratories), since *C. jejuni* does not grow well in Mueller-Hinton broth. The brucella broth cultures of *C. jejuni* were incubated for 18 to 24 h (incubation time required for the slow-growing *C. jejuni* to show visible growth in broth). This culture was then diluted in brucella broth until it contained approximately 10^7 organisms per ml by comparison with a visual turbidity standard.

The diluted culture broths were applied to the surface of the antibiotic agar plates with a Steers replicator (13). The plates were incubated for 48 h at 42°C in an atmosphere containing 5% O_2. MICs were recorded as the lowest TMP concentration that allowed no growth.

Plate counts. Twenty-four-hour cultures of *C. jejuni* in brucella broth were diluted until the concentration approximated 10^8 organisms per ml. After incubation for 24 h, Mueller-Hinton broth cultures of the control strains were diluted similarly. These broth cultures were diluted out to 10^{-7} in a series of 10-fold dilutions. A 0.1-ml quantity of each dilution was inoculated to duplicate plates of each medium and spread over the surface of the agar. After incubation for 18 to 24 h, the total number of colonies was recorded.

RESULTS

TMP was not active against the four TMP-sensitive control strains when tested in Skirrow medium (blood agar base no. 2 and 7% lysed horse blood) or in Campy-BAP medium (brucella agar and 10% sheep blood). As expected, the MICs of TMP were low when strains were tested in Mueller-Hinton medium with either 7% lysed horse blood or 10% sheep blood (Table 1). Thymidine phosphorylase, at the manufacturer's recommended concentration of 0.1 U/ml (recommended for use in media with moderate levels of thymidine), was also ineffective in restoring the activity of TMP in Skirrow or Campy-BAP base (Table 2). In these two basal media with 0.1 U of thymidine phosphorylase per ml, the MICs of TMP for the four control organisms were all greater than 32 μg/ml, compared with 1 μg/ml or less in Mueller-Hinton.

Swenson and Thornsberry (12) found that 0.3 μg of thymidine per ml in Mueller-Hinton broth inhibited the action of sulfamethoxazole-TMP for all of the organisms they tested. It was therefore no surprise that when 0.3 μg thymidine per ml was added to Mueller-Hinton medium, the MICs of TMP for *E. coli, Staphylococcus aureus*, and *Streptococcus faecalis* were all greater than 32 μg/ml (Table 3). The addition of lysed horse blood or thymidine phosphorylase to Mueller-Hinton medium with thymidine restored the activity of TMP against *E. coli* and *Staphylococcus aureus*, but the MIC for *Streptococcus faecalis* was greater than 32 μg/ml

TABLE 1. MICs of TMP in basal media (Skirrow and Campy-BAP)

Strain	MIC (μg/ml) in:			
	Skirrow base[a]	Mueller-Hinton + 7% lysed horse blood	Campy-BAP base[b]	Mueller-Hinton + 10% sheep blood
P. mirabilis	>128	2	>128	2
Streptococcus faecalis	>128	0.25	>128	2
Staphylococcus aureus	>128	≤0.25	>128	≤0.25
E. coli	128	0.5	>128	2
C. jejuni A1446	>128	>128	>128	>128
C. jejuni A1256	>128	>128	>128	>128
C. jejuni E6484	>128	>128	>128	>128
C. jejuni NCTC 11168	>128	>128	>128	>128

[a] Blood agar base no. 2 (Oxoid) and 7% lysed horse blood.
[b] Brucella agar (BBL) and 10% sheep blood.

TABLE 2. Addition of thymidine phosphorylase and lysed horse blood to basal media

Strain	TMP MIC (μg/ml) in:[a]						
	Mueller-Hinton	Blood agar base no. 2	Blood agar base no. 2 + TP	Blood agar base no. 2 + 7% LHB	Brucella agar	Brucella agar + TP	Brucella agar + 7% LHB
Streptococcus faecalis ATCC 29212	≤0.25	>32	>32	>32	>32	>32	>32
Staphylococcus aureus ATCC 25923	1	>32	>32	>32	>32	>32	>32
E. coli ATCC 25922	1	>32	>32	>32	>32	>32	>32
C. jejuni NCTC 11168	>32	>32	>32	>32	>32	>32	>32

[a] TP, Thymidine phosphorylase (0.1 U/ml of medium); LHB, lysed horse blood. Blood agar base no. 2 is the basal agar for Skirrow medium; brucella agar is the basal agar for Campy-BAP medium.

even with thymidine phosphorylase or lysed horse blood in the medium.

Tables 4 and 5 show the results of total plate counts of three sensitive control organisms (*E. coli, Streptococcus faecalis*, and *P. mirabilis*) on the two basal media (Campy-BAP and Skirrow base) and on Mueller-Hinton broth with 10% sheep blood and 7% lysed horse blood. Each of these four media was tested without antimicrobial agents and with three different combinations of antimicrobial agents: TMP alone, all agents except TMP, and all agents including TMP.

E. coli showed no sensitivity to TMP on Campy-BAP or Skirrow base with TMP, but had a 7-log reduction in count on Mueller-Hinton with 10% sheep blood and TMP and an 8-log reduction in count on Mueller-Hinton with 7% lysed horse blood and TMP. However, although the *E. coli* strain was not affected by TMP alone in Campy-BAP or Skirrow base, complete Campy-BAP and Skirrow media were more inhibitory for *E. coli* than were those media without TMP.

Streptococcus faecalis was not sensitive to TMP in Campy-BAP or Skirrow base, but was sensitive in Mueller-Hinton with sheep blood and TMP and in Mueller-Hinton with horse blood and TMP. It was not possible to assess whether there was additional inhibition of *Streptococcus faecalis* when TMP was included in Campy-BAP or Skirrow medium, since those two media without TMP both completely inhibited the organism.

P. mirabilis showed interesting results in that it was inhibited by TMP alone in Campy-BAP or Skirrow base by 2 logs. *P. mirabilis* had counts of zero on Campy-BAP base with vancomycin, polymyxin B, amphotericin B, and cephalothin, and therefore the effect of the addition of TMP was undetermined. *P. mirabilis* showed very little inhibition when tested on Skirrow base with vancomycin and polymyxin B, but its counts were reduced by almost 4 logs when TMP was included with those two antibiotics.

DISCUSSION

We found that TMP was inactive against *E. coli* and *Streptococcus faecalis* when used as the only antimicrobial agent in the agar base media Campy-BAP and Skirrow. It is not surprising that these two nutritionally rich media, blood agar base and brucella agar, may contain sufficient thymidine to interfere with the activity of TMP. Concentrations of thymidine greater than 0.03 μg/ml in media have been found to increase

TABLE 3. Reversal of thymidine inhibition of TMP activity by lysed horse blood and thymidine phosphorylase as measured by MICs

Strain	TMP MIC (μg/ml) in:[a]			
	MH	MH + thymidine	MH + thymidine + LHB	MH + thymidine + TP
C. jejuni E6484	>32	>32	>32	>32
E. coli ATCC 25922	0.5	>32	0.5	0.5
Staphylococcus aureus ATCC 25923	1.0	>32	0.5	1.0
Streptococcus faecalis ATCC 29212	<0.25	>32	>32	>32

[a] MH, Mueller-Hinton medium; LHB, 7% lysed horse blood; TP, thymidine phosphorylase (0.1 U/ml). Thymidine concentration, 0.3 μg/ml.

TABLE 4. Yield of sensitive control strains on Campy-BAP and modified Campy-BAP media

Medium and drugs[a]	Plate count (no. of colonies)		
	E. coli	*Streptococcus faecalis*	*P. mirabilis*
Campy-BAP Base[b]			
None	3.8×10^8	2.0×10^8	3.2×10^8
TMP	3.7×10^8	2.0×10^8	2.8×10^6
TMP, Van, Poly B, AmB, Ceph	2.0×10^1	0	0
Van, Poly B, AmB, Ceph	1.1×10^3	0	0
Mueller-Hinton + 10% Sheep Blood			
None	3.2×10^8	1.9×10^8	2.3×10^8
TMP	3.1×10^1	1.0×10^1	2.1×10^3
TMP, Van, Poly B, AmB, Ceph	0	0	0
Van, Poly B, AmB, Ceph	7.5×10^2	0	0

[a] Abbreviations for and concentrations of drugs (per liter): Van, vancomycin, 10 mg; Poly B, polymyxin B, 2,500 IU; AmB, amphotericin B, 2 mg; and Ceph, cephalothin, 15 mg. TMP was added at 5 mg/liter.
[b] Brucella agar and 10% sheep blood.

MIC values (3). When MICs were determined, we found that 0.3 μg of thymidine per ml added to Mueller-Hinton agar completely inactivated the TMP. It is also not surprising that thymidine phosphorylase or lysed horse blood (which contains thymidine phosphorylase) failed to make these media suitable for susceptibility testing with TMP. Ferone et al. (5), who identified the active factor in lysed horse blood as thymidine phosphorylase, found that it could not overcome the reversal of TMP activity in media that contain levels of thymidine greater than 10 μg/ml. This could be due to either the presence of uncleaved thymidine or the possibility that high levels of thymine can be utilized in place of thymidine. *Streptococcus faecalis* can utilize thymine as well as thymidine, and thus the MIC of TMP was high in the presence of a small amount of thymidine which should have been neutralized by the thymidine phosphorylase and lysed horse blood added (5). It is possible that levels of thymidine phosphorylase higher than those used in this study would lower the thymidine levels in these two media, but it would also make them more expensive and probably would not make them more inhibitory for *Streptococcus faecalis*.

It is interesting that in these media, which apparently have sufficient thymidine to affect the activity of TMP, the activity of the other antimicrobial agents was enhanced by TMP. It seems likely that this increased activity is due to a synergistic mechanism and, in fact, synergism between TMP and polymyxin has been reported (10). The polymyxins and TMP have synergistic activity against some *Proteus* species which are resistant to each drug individually (10). Garrod and Waterworth (6) showed that the combination of TMP and polymyxin B is bactericidal for *P. mirabilis*, although TMP by itself is only bacteriostatic and polymyxin B alone is ineffective.

TABLE 5. Yield of sensitive control strains on Skirrow and modified Skirrow media

Medium and drugs[a]	Plate count (no. of colonies)		
	E. coli	*Streptococcus faecalis*	*P. mirabilis*
Skirrow base[b]			
None	3.4×10^8	2.5×10^8	5.1×10^8
TMP	2.9×10^8	1.5×10^8	2.1×10^6
TMP, Van, Poly B	9.5×10^1	0	2.0×10^4
Van, Poly B	4.5×10^3	0	1.5×10^8
Mueller-Hinton + 7% horse blood			
None	3.0×10^8	1.7×10^8	3.6×10^8
TMP	0	0	8.0×10^2
TMP, Van, Poly B	0	0	1.8×10^2
Van, Poly B	2.7×10^3	0	9.3×10^7

[a] See Table 4, footnote *a*, for drug abbreviations and concentrations.
[b] Blood agar base no. 2 and 7% lysed horse blood.

In conclusion, TMP alone is not active in the basal agars of two media selective for *C. jejuni*, Skirrow medium and Campy-BAP, probably because they contain high levels of thymidine. TMP is active in the presence of the other antimicrobial agents in these selective media, which is possibly due to synergism between TMP and polymyxin B. For this reason, it appears that lysed horse blood is not required for selective media containing TMP and other antimicrobial agents. This could be useful for laboratories which are using Skirrow medium but have no source of horse blood. Sheep blood (or another readily available kind of blood) could probably be substituted without compromising the selectivity of the medium. Since our experiments were performed with multiply sensitive quality control strains, any selective medium containing TMP without lysed horse blood or thymidine phosphorylase should be evaluated with routine clinical specimens.

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