Prediction of Sulfamethoxazole-Trimethoprim Synergistic Action Against Members of the Family *Enterobacteriaceae* with a Two-Plate Agar Dilution Breakpoint MIC System

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Synergy between sulfamethoxazole (SMZ) and trimethoprim (TMP) was predicted by a two-plate agar dilution breakpoint MIC system. Comparison of the results of this new system with those of the disk diffusion system (P. M. Waterworth, *Postgrad. Med. J. Suppl.* 45:21-27, 1969) after tests with 1,518 *Enterobacteriaceae* isolates showed an overall correlation of 99.8%, a sensitivity of 99.7%, and a specificity of 100%. The method involves spot inoculation of 10^3 organisms onto each of two plates, one containing 160 μg of SMZ per ml and the other 8 μg of TMP per ml (in Oxoid IsoSensitest medium with 3% agar supplemented with 7% saponin-lysed horse blood), and then incubation overnight at 37°C in air. All but three organisms for which SMZ-TMP was found to be synergistic by disk testing were inhibited on both plates. Three isolates of *Proteus mirabilis*, which failed to correlate with disk testing by this new system, all showed SMZ MICs of 1,000 $\mu\text{g}/\text{ml}$. The SMZ-TMP combination was falsely predicted to be nonsynergistic against these three organisms. There were no false synergy predictions by the breakpoint MIC system. Laboratories should report susceptibility to the SMZ-TMP combination only when there is synergy between the constituents. This simple, reliable agar dilution technique enables laboratories to accurately report synergy between SMZ and TMP.

Co-trimoxazole, a combination of sulfamethoxazole (SMZ) and trimethoprim (TMP), is the treatment of choice for many urinary and respiratory tract infections (3) and for many other infections, including nocardiosis (11, 13).

The methods most commonly used in laboratories for SMZ-TMP susceptibility testing determine susceptibility to these agents in combination (9). This practice has been questioned (4) since it may only represent susceptibility to one of the agents. It has been proposed that laboratories should report susceptibility to co-trimoxazole only when synergy between the components has been demonstrated (2). The recognized techniques for demonstrating synergy between SMZ and TMP include full checkerboard combination, empiric dose-response curves (6), and three-disk disk diffusion testing (5). However, these techniques are cumbersome, particularly for laboratories which use an agar dilution susceptibility testing system.

The purpose of this investigation was to develop a simple, reliable agar dilution testing system to predict synergy between SMZ and TMP. The method was based on the observation that when high-level resistance was present for one or both components, synergy was not demonstrable. Others have previously suggested that high-level resistance might correlate with lack of synergy (1, 12). However, no previous study has examined large numbers of *Enterobacteriaceae* and correlated high-breakpoint MICs of SMZ and TMP with the results of the three-disk testing system.

MATERIALS AND METHODS

Bacteria. A total of 1,518 routine laboratory isolates of the family *Enterobacteriaceae* cultured from urine (85%), sputum (9%), and wound swabs (6%) were tested in the comparison studies. Of these organisms, the first 562 had MICs determined for SMZ, TMP, and a combination of SMZ and TMP at a ratio of 20:1.

Antimicrobial agents. SMZ and TMP lactate were obtained as powders of known purity from the Wellcome Foundation Ltd. SMZ was dissolved in methanol to a concentration of 100 mg/ml and then diluted in water to give a stock solution of 16 mg/ml. TMP lactate was dissolved in water to give a final concentration of 800 $\mu\text{g}/\text{ml}$ as a stock solution. Stock solutions were stored at -20°C until used. Sensitivity disks (Oxoid Ltd.) contained 25 μg of SMZ or 1.25 μg of TMP; the combination disk contained 23.75 μg of SMZ plus 1.25 μg of TMP.

MIC susceptibility testing. MICs of each agent were determined for the first 562 *Enterobacteriaceae* isolates by an agar dilution method. IsoSensitest (Oxoid) containing 3% agar, supplemented by the addition of 7% saponin-lysed horse blood, was used since this medium has been found to be noninhibitory to the action of sulfonamides (5). Plates (9 cm) containing 20 ml of agar were poured at least 1 day before the experiment to allow time for inhibitory components to be inactivated (5). Antibiotic concentrations in these plates, in serial doubling dilutions, ranged from 160 to 1.2 $\mu\text{g}/\text{ml}$ for SMZ, from 8 to 0.06 $\mu\text{g}/\text{ml}$ for TMP, and from 80:4 to 0.6:0.03 $\mu\text{g}/\text{ml}$ for the SMZ-TMP combination. The plates were surface dried for 4 to 6 h at 25°C before inoculation with a saline suspension of test organisms to deliver 10^3 CFU per spot, using a Steers type replicator. This low inoculum was chosen to avoid an inoculum effect when testing SMZ (5). After overnight incubation at 37°C in air, the plates were observed for growth or no growth by using transmitted light; a faint haze in comparison with the control was recorded as no growth (3).

Disk diffusion susceptibility testing. IsoSensitest agar (17 ml) supplemented with 4% saponin-lysed horse blood was poured into a 9-cm-diameter petri dish to a depth of 4 mm. A cotton swab dipped into a log-phase broth culture of the test organism standardized to a McFarland 0.5 turbidity standard was wiped across the diameter of a plate, and a sterile swab was used to spread the inoculum with a rotary inoculator.

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TABLE 1. Results of testing 1,518 *Enterobacteriaceae* isolates and interpretation of breakpoint MICs to predict synergy between SMZ and TMP^a

Growth against:		Prediction	No. (%)
SMZ (160 µg/ml)	TMP (8 µg/ml)		
—	—	Synergy	1,069 (70.5)
+	—	No synergy	201 (13.2)
—	+	No synergy	41 (2.7)
+	+	No synergy	207 (13.6)

^a The breakpoint MIC prediction system showed an overall correlation with the disk diffusion system of 99.8%.

After overnight incubation, this method consistently produced the "just not confluent" growth recommended for SMZ-TMP susceptibility testing (5).

Determination of synergy. (i) Disk method. Antibiotic disks containing 25 µg of SMZ, 1.25 µg of TMP, and a combination of 23.75 µg of SMZ plus 1.25 µg of TMP were placed equidistant, 50 mm apart, in a triangular arrangement on the plate. After incubation for 18 h at 37°C in air, the diameters of the zones of inhibition were measured and compared. The combination was interpreted to be synergistic by disk testing if the zone around the SMZ-TMP combined disk was ≥16 mm and was also larger than each of the zones around SMZ or TMP tested alone (5).

(ii) MIC method. For evaluation of synergistic activity against 562 *Enterobacteriaceae* isolates with a series of agar dilution plates, the fractional inhibitory concentration index (FIC index) was calculated as follows: FIC index = (MIC of SMZ combination/MIC of SMZ alone) + (MIC of TMP combination/MIC of TMP alone).

Any value of the FIC index ≤0.5 was considered to indicate synergy (6). The FIC index was not calculated when the MIC for either drug was at the lowest concentration tested for that drug.

RESULTS

Examination of the results of testing 562 *Enterobacteriaceae* isolates for synergy between SMZ and TMP by the three-disk system (5) and the agar dilution system showed that synergy could not be demonstrated with either system when an organism displayed high-level resistance to one or both components, i.e., SMZ MIC > 160 µg/ml or TMP MIC > 8 µg/ml or both (see Table 1). Conversely, for organisms with SMZ MICs ≤ 160 µg/ml and TMP MICs ≤ 8 µg/ml, synergy was always seen on disk testing.

An additional 956 *Enterobacteriaceae* isolates were tested for SMZ or TMP high-level resistance (or both), and results were compared with synergy determination by the three-disk system.

Table 1 explains how the high-level MIC determinations were interpreted to predict synergy, or nonsynergy, between SMZ and TMP. A correlation of 99.8% between the three-disk method and the high-level breakpoint MIC prediction was found for the 1,518 *Enterobacteriaceae* isolates tested. Synergy was predicted between SMZ and TMP on the basis of breakpoint MICs in 1,069 (70.5%) of the organisms tested when SMZ MIC ≤ 160 µg/ml and TMP MIC ≤ 8 µg/ml; in all instances, disk testing confirmed these predictions. Of the test isolates, 449 (29.5%) had MICs in excess of one or both of these values (Table 1), and, as predicted, no synergy could be demonstrated on disk testing, except for 3 of 149 *Proteus mirabilis* isolates. Further testing of these three

isolates confirmed synergy with an FIC index of 0.5. Of 1,072 predictions of synergy by the disk diffusion method, 3 of these were predicted to show no synergy by the breakpoint MIC method. All 446 predictions of no synergy by the disk diffusion method agreed with the predictions by the breakpoint MIC method. The MICs of SMZ alone were ca. 1,000 µg/ml, and MICs of TMP alone were 4 µg/ml, whereas in combination the MICs were 250 and 1 µg/ml, respectively.

Synergy between SMZ and TMP was not demonstrable by three-disk testing for 446 of 1,518 isolates, but 219 of these 446 no-synergy isolates showed a zone of ≥16 mm on single combination disk testing. A zone of ≥16 mm is claimed by the advocates of single disk testing to equate with synergy (5).

DISCUSSION

The introduction of the SMZ-TMP combination brought with it a debate, which still continues, regarding susceptibility testing methodology, interpretation of results (particularly with respect to the synergistic activity of this drug combination), and the relevance of the laboratory findings to the clinical situation (4).

The relatively recent release of TMP for use as a single agent, together with reports of increases in MICs of TMP or SMZ or both (4, 10), highlight the need for review of susceptibility testing and reporting procedures (2).

The most common methodology currently used is based on original work by both Bushby and Waterworth, who demonstrated that synergy between SMZ and TMP could be determined by the three-disk method (5, 15). However, they recommended that a single SMZ-TMP disk could be used to determine susceptibility. This recommendation has been taken up by the National Committee of Clinical Laboratory Standards (NCCLS) (9) and also by Barry and Thornsberry (3). Although these methods are incapable of demonstrating synergy, Washington and Sutter, as well as NCCLS, have further extended the approach into agar dilution MIC systems by testing susceptibility to the combination (8, 14).

Lewis et al. (7), in reviewing SMZ-TMP therapy, suggested that the SMZ-TMP combination offers no enhanced efficacy over TMP alone. However, the increasing MICs of SMZ and TMP over the last decade have resulted in renewed interest in the clinical relevance of synergy (4, 12). Reeves has recently reported organisms showing "resistance" to SMZ and TMP in which synergy of the combination could still be demonstrated (12).

O'Brien et al. believe that the SMZ-TMP combination can offer therapeutic advantages over TMP alone in treating some clinical infections (10). Amyes et al. suggest that it would be timely to review the way laboratories test and report SMZ-TMP susceptibility (2). When an organism had high-level resistance to one or another of the components, synergy could not be demonstrated with the combination, and it would be expected that concurrent use of these drugs would have no therapeutic advantage over therapy with the active agent alone in these instances (Table 1). Indeed, the potential toxic effects of an antimicrobial agent, particularly SMZ, should be avoided if an agent has no enhancing activity in a drug combination. Laboratories should, therefore, report susceptibility to a drug combination such as SMZ-TMP only when synergy between both components can be demonstrated or predicted. This approach should discourage unnecessary use of SMZ-TMP in combination unless they are synergistic.

The results of testing 1,518 *Enterobacteriaceae* isolates by the three-disk method compared with a single combination

disk confirmed that it is not possible to predict synergy with a single combination disk. If susceptibility to a combination disk had been equated with synergy, then 219 (49%) of 446 isolates (for which the SMZ-TMP combination was found nonsynergistic on three-disk testing) would have been inappropriately assessed. On the basis of single combination disk testing, it is not possible to know whether any observed effect is due to the action of either drug acting alone or to both drugs acting synergistically. Each single SMZ-TMP combination plate used was found as unreliable a predictor of synergy as a single combination disk. The reporting of susceptibility based on single combination disk or single combination plate testing would have encouraged the inappropriate prescription of a potentially toxic and microbiologically inactive drug for ca. 1 in 6 patients studied in this series.

The only disk method capable of reliably detecting synergy is the three-disk method of Bushby. An alternative to this rather cumbersome method was developed in our laboratory, which predicts synergy by means of a two-plate high-level breakpoint MIC system, the interpretation of which is shown in Table 1. This simple, reliable system allows the laboratory to report susceptibility to the SMZ-TMP combination only when synergy between both components is present. High sensitivity (99.7%) and specificity (100%) was calculated when results of testing 1,518 *Enterobacteriaceae* isolates were compared with results of the three-disk disk diffusion system.

The validity of the high-breakpoint MIC method has not yet been tested for gram-positive cocci or fastidious organisms. A prerequisite for extension of this system into gram-positive cocci would be calibration of appropriate breakpoint MICs against the three-disk method.

In conclusion, it is emphasized that the concentrations of SMZ and TMP used in the high-level breakpoint MIC synergy prediction method described in this paper are not appropriate for the determination of microbial sensitivity to these agents if they are to be administered to a patient singly.

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