In Vitro Sensitivity of *Salmonella* to Ten Antimicrobial Agents Including Sulfamethoxazole and Trimethoprim, Alone and in Combination

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The activities of trimethoprim (TMP) and sulfamethoxazole (SMZ), alone and in combination (SMZ-TMP), and of the following antibiotics were tested against 115 clinical isolates of nontyphoid *Salmonella* species: tobramycin, gentamicin, ampicillin, amoxicillin, neomycin, kanamycin, chloramphenicol, and tetracycline. The methods of disk diffusion, microtiter broth dilution, and agar dilution were employed for all single antimicrobial agents as well as for SMZ-TMP studies. Growth curves were performed in broth. SMZ-TMP, TMP, gentamicin, tobramycin, and neomycin were the most active drugs in vitro. All strains were inhibited by ≤1 μg of TMP per ml, but >100 μg of SMZ per ml was required for at least 10% of strains. SMZ and TMP in a ratio of 10:0.5, respectively, inhibited all isolates and were synergistic for 105 strains. All strains inhibited by the combination of 10:0.5 SMZ-TMP had a zone diameter of ≥22 mm by using a combination disk containing 1.25 μg of TMP and 23.75 μg of SMZ. Seven isolates were resistant to >100 μg/ml of ampicillin or amoxicillin; all isolates were sensitive to chloramphenicol at ≤6.3 μg/ml. SMZ-TMP appears to be active against nontyphoid salmonellae in vitro; this is usually due to a synergistic effect.

The combination of sulfamethoxazole-trimethoprim (SMZ-TMP) has demonstrated activity in vitro (5, 6, 7, 15) and in vivo (1, 4, 9-11, 16, 18) against typhoid and nontyphoid *Salmonella* strains. The mechanism of action of this combination (inhibition of bacterial folate metabolism) is thought to be responsible for the antibacterial synergy often demonstrated against gram-negative bacteria (7, 17). A study was designed to investigate the in vitro activities of SMZ and TMP, alone and in combination, against clinical isolates of *Salmonella* and to compare these activities with the following antibiotics: tobramycin, gentamicin, amoxicillin, ampicillin, kanamycin, neomycin, chloramphenicol, and tetracycline.

**MATERIALS AND METHODS**

One hundred and fifteen recent clinical isolates of nontyphoid salmonellae, isolated from 1 June 1971 to 30 June 1972, were identified according to standard methods in use at the Montreal Children’s Hospital microbiology laboratory. The isolates were from the stools or blood, or both, of patients with gastroenteritis (98 patients) or asymptomatic family contacts (17 patients) and consisted of the following serotypes: 43 *S. typhimurium*, 19 *S. montevideo*, 11 *S. heidelberg*, 9 *S. thompson*, 7 *S. infantis*, 6 *S. enteritidis*, 5 *S. saintpaul*, 5 *S. blockley*, 3 *S. shwarzenberg*, 2 *S. braenderup*, 2 *S. muenchen*, 1 *S. javiana*, 1 *S. poona*, and 1 *S. java*. Disk diffusion sensitivities were performed on Mueller-Hinton (M-H) agar by a modification of the Kirby-Bauer method (2). Disk potencies were as follows in micrograms: gentamicin, 10; kanamycin, 30; neomycin, 30; chloramphenicol, 30; tetracycline, 30; ampicillin, 10; amoxicillin, 10; tobramycin, 10; sulfathiazole, 300; sulfamethoxazole, 23.75; trimethoprim, 1.25; and sulfa-tetra- trimethoprim (combination disk; 23.75 μg of sulfamethoxazole plus 1.25 μg of trimethoprim).

Antimicrobial agents were supplied in pure powders for broth and agar dilution tests: gentamicin (Schering lot no. GMC-IM-208), kanamycin (Bristol no. 71F379), chloramphenicol (Parke, Davis no. 7172F), neomycin (Upjohn no. R1335), tetracycline (Bristol no. 70F1628), ampicillin (Ayerst no. 2495-PF), amoxicillin (Ayerst no. 2577-QF), tobramycin (Lilly no. XU4911 Enx), sulfamethoxazole...
Broth dilution minimum inhibitory concentrations (MICs) were performed by a microtiter method in M-H broth, except for SMZ and TMP which were performed in Trypticase soy broth (12). MICs were read after approximately 18 h of incubation at 37°C and were defined as the lowest concentration of antimicrobial agent in which no growth was visible except for SMZ and TMP where MIC was defined as the lowest concentration of drug in which there was a 50% reduction of growth as judged by comparison with a standard control tube. These criteria were employed for SMZ and TMP because gradually decreasing turbidity, rather than abrupt changes in visible growth, were encountered with these drugs. The SMZ-TMP combination broth studies were performed by the microtiter checker-board method. All values represent end concentrations (actual concentration of drug in each tube after the addition of all reagents) in these tests.

Agar dilution MICs were performed as previously described (19) by using M-H agar and an inoculum of a 10⁻³ dilution of an overnight broth culture of Salmonella. MIC was defined as the lowest concentration of drug which exhibited no growth except for SMZ where MIC was defined as the lowest concentration of drug inhibiting confluent growth of large colonies. Combination studies were performed as previously described (8), and values represent end concentrations: end points represented no growth. Representative isobolograms were constructed for the broth and agar dilution studies and synergism was defined as previously described (8). The concentrations of each drug pair are plotted on an arithmetic scale on the ordinate and abscissa. Each plotted point represents the same biological activity, i.e., the minimum amount of one drug in the presence of various concentrations of the other necessary to inhibit growth. The line joining the points, the isobole, thus represents a continuum of equal antibacterial effect of a pair of drugs. If an isobole connecting the MIC of each drug acting independently follows a straight line, the combined effect is additive; if the isobole is concave (bows in), the combined effect is synergistic; and it it is convex (bows out), the combined effect is antagonistic.

Growth curves were performed on two strains of Salmonella in Trypticase soy broth with 4% lysed horse blood added. A 0.05-ml portion of an overnight broth culture of each organism was introduced into a 250-ml flask containing 100 ml of Trypticase soy broth and 4% lysed horse blood and incubated on a rotary shaker at 37°C. Samples were taken at 0, 1, 2, 3, 4, 5, and 6 h after the beginning of incubation, and colony counts were made from appropriate dilutions.

RESULTS

One hundred and fifteen isolates of Salmonella were tested against 10 antimicrobial agents by the disk diffusion method (3). All were sensitive to gentamicin, tobramycin (zone ≥18 mm), kanamycin, neomycin, and chloramphenicol. Seven strains were resistant to each of ampicillin, amoxicillin (zone ≥19 mm), and sulfathiazole (300-mg disk). Only five strains were sensitive to tetracycline, the rest being intermediate (87%) or resistant (8.5%). The zone diameters obtained with the 23.75-µg SMZ and the 1.25-µg TMP disks alone, and in combination, are plotted against the agar dilution MICs in Fig. 1 to 3. Although the TMP disk zone diameters correlated well with broth and agar dilution MICs (Fig. 1), the SMZ disk results did not (Fig. 2). By using Bushby's criteria (S. R. M. Bushby, paper presented at the Trimethoprim-Sulphonamide Conference, Boston, Dec. 1972) of 14 mm or greater for sensitive strains there is a reasonable correlation of zone diameters with the SMZ-TMP combination and the agar dilution MICs obtained (Fig. 3). The disk zone diameters for SMZ were often indistinct because of gradually fading inner zones consisting of smaller colonies; zone diameters were measured, therefore, by using the more distinct larger outer zone in all cases. The addition of 4% lysed horse blood to M-H agar did not appreciably change the disk diffusion results (plates used immediately or after overnight storage at 4°C). There was good correlation between disk diffusion zone diameters and the agar and broth dilution MICs for the other antimicrobial agents tested by using standard Kirby-Bauer zone diameter criteria. Amoxicillin and ampicillin exhibited cross resistance for seven isolates.

M-H broth was found to be inappropriate for the SMZ and TMP broth dilution studies for end points were often indistinct and not reproducible. Trypticase soy broth was better; however, a 50% inhibition (compared with controls) end point was chosen in the TMP and SMZ tests for there was a gradual decrease in growth seen throughout the wells. The addition of 4% lysed horse blood often lowered the MIC approximately two to four dilutions and allowed for easier interpretations of end points for TMP. This allows definition of broth dilution MICs for TMP as the lowest drug concentration inhibiting visible growth. Synergism was demonstrated for 105 of the 115 strains of Salmonella by using the broth dilution method. These results were confirmed by growth curves for two isolates of Salmonella. Synergism was marked with both SMZ-resistant (Fig. 4) and SMZ-sensitive (12.5 µg/ml) strains (TMP MIC for both strains was 0.6 µg/ml).

Agar dilution studies on 115 isolates of Salmonella are demonstrated in Fig. 5. Trimethoprim, gentamicin, and tobramycin were the most active agents. The average MIC for
amoxicillin was 1.84 µg/ml and for ampicillin was 2.99 µg/ml; cross-resistance was demonstrated for seven isolates. All strains were inhibited by ≤6.3 µg of chloramphenicol per ml. Although 50% of strains were inhibited by ≤1.6 µg of tetracycline per ml, 10% of strains required ≥3.1 µg/ml and 5% ≥50 µg/ml. The addition of 4% lysed horse blood to M-H agar did not appreciably change the end points for the SMZ and TMP studies. The cumulative percentages of *Salmonella* isolates inhibited by SMZ and TMP alone and in combination at a ratio of 20:1 are represented in Fig. 6. Combining the drugs in a 3:1 ratio was also effective. Combinations of SMZ-TMP were more active than either drug alone; at high concentrations the differences in activities of the combination and TMP alone became less marked. That this represents a synergistic action in most instances was shown by the growth curves mentioned above and by combination studies in broth and M-H agar. The latter tests demonstrated a synergy for 105 of the 115 strains. A composite isobologram for these 105 strains is represented in Fig. 7 by using the geometric mean concentrations for the 105 strains.
was noted for the one strain of *Salmonella typhi* studied. Pugsley et al. (15) found 49 out of 50 *Salmonella typhi* were sensitive to trimethoprim at ≤0.12 µg/ml and to sulfamethoxazole at ≤32 × µg/ml; synergism was present for 21 of the 50 strains studied with the drugs in a 10:1 ratio (SMZ-TMP). Our data show that all 115 nontyphoidal *Salmonella* were inhibited by ≤1 µg of TMP per ml, that 90% were inhibited by ≤100 µg of SMZ per ml, and that synergism was present for 91% of strains when the drugs were combined in their usual serum ratio of 20:1 (SMZ-TMP).

The reliability of the disk diffusion method in predicting these sensitivities in vitro is confirmed for the *Salmonella* isolates and all of the antimicrobial agents tested except for the 23.75-µg SMZ disk. A disk zone diameter of ≥23 mm for the SMZ-TMP combination was correlated with sensitivities of all isolates to <10:0.5 µg of SMZ-TMP per ml.

Synergy was demonstrated in vitro for the combination SMZ-TMP against most of the strains of *Salmonella*, and this was confirmed by growth curves in two representative studies. Previous pharmacokinetic studies have demonstrated serum ratios of approximately 20:1 and urine ratios of 3:1 for SMZ-TMP (M. I. Marks et al., manuscript submitted for publication).

Our data indicate that the drugs are active against *Salmonella* in vitro in both ratios. This was true for both SMZ-sensitive and -resistant strains.

The technical difficulties of sensitivity testing for SMZ-TMP are well illustrated by the lack of clear 100% inhibition end points in broth and the necessity for the addition of 4% lysed horse blood for these studies. Horse blood is necessary to neutralize thymine and thymidine which inhibits the activity of trimethoprim (6, 12). The lack of such an effect in M-H agar is probably due to the low thymidine content of this material; this may vary in different lots. The effects of media on SMZ-TMP sensitivity testing have been described in detail by Bushby (5) and Waterworth (21). Our data indicate that Trypticase soy broth with 4% lysed horse blood is reliable for broth dilution and that M-H agar (without blood) is reliable for agar dilution studies with SMZ, TMP, and SMZ-TMP.

There is a significant difference between in vitro sensitivities and in vivo results in the treatment of *Salmonella* infections in humans. Although highly active in vitro against nontyphoidal *Salmonella*, further clinical evaluation is necessary before recommendations can be made regarding the usefulness of SMZ-TMP compared with established drugs such as ampicillin.

**DISCUSSION**

This study has confirmed the sensitivity in vitro of nontyphoidal *Salmonella* isolates to a variety of antimicrobial agents including SMZ-TMP, gentamicin, tobramycin, amoxicillin, and ampicillin. Tobramycin and amoxicillin closely reflect the sensitivities in vitro exhibited by gentamicin and ampicillin, respectively. The results in this study parallel those by Meyers and Hirschman (14) and Sutherland et al. (20) for tobramycin and amoxicillin, respectively, and those of Darrell et al. (7), Bushby and Hitchings (6), and Pugsley et al. (15) for SMZ and TMP.

Darrell et al. (7) studied 14 strains of *Salmonella* and found a two- to fourfold increase in the activity of trimethoprim in the presence of sulfafurazol in a ratio of 9:1 (sulfafurazol-trimethoprim). Bushby (5) reported that the sensitivities of four *Salmonella* strains were 0.1 to 0.4 µg/ml for trimethoprim and 4 to 30 µg/ml for sulfadiazine; synergism
and chloramphenicol in the treatment of *Salmonella* infections in humans. Based on in vitro data, amoxicillin, gentamicin, and tobramycin may also be useful in the treatment of *Salmonella* infections.

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**LITERATURE CITED**