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Co-Trimoxazole Susceptibility Tests Improved with Separate Trimethoprim and Sulfamethoxazole Disks

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It is impossible to test accurately bacterial susceptibility to the trimethoprim-sulfamethoxazole combination co-trimoxazole with a single combined susceptibility disk. However, a variety of factors still affect the result even when separate trimethoprim and sulfamethoxazole disks are used. Experiments with separate disks showed that the optimum conditions for testing the susceptibilities of enterobacteria to these drugs were to flood-seed an agar plate with an inoculum of 10^4 to 10^5 organisms per ml, take off the excess liquid, and place a disk of 1 μg of trimethoprim and another of 50 μg of sulfamethoxazole on the surface of the agar with their centers exactly 25 mm apart. This method not only allowed the determination of resistance but also distinguished synergy.

The agar diffusion technique is the method most commonly used to detect bacterial susceptibility to the antibacterial combination co-trimoxazole. Most laboratories determine bacterial susceptibility to the two component drugs of the combination, trimethoprim and sulfamethoxazole, by using a single disk. This disk usually contains 1 part trimethoprim and 20 parts sulfamethoxazole. However, the use of one disk for the susceptibility testing of two drugs is contrary to the guidelines of the Expert Committee on Antibiotics of the World Health Organization (10). This committee proposed that two drugs should never be tested with a single disk, because it is impossible to determine the resistance patterns to the individual drugs.

Trimethoprim, until recently, has always been marketed in conjunction with sulfamethoxazole in order to exploit the synergy between the two drugs (7). Any susceptibility test with a single disk containing both of these drugs automatically incorporates the synergy between them. Under these circumstances, it is impossible to take account of this interaction. This paper investigates the optimum conditions for showing the susceptibilities to the individual drugs and also allowing the demonstration of synergy.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria used were *Escherichia coli* 114, a prototrophic strain susceptible to trimethoprim and sulfamethoxazole, obtained from the postmortem of a child (1). *E. coli* R is a clinically isolated strain that is resistant to both trimethoprim and sulfamethoxazole. *E. coli* 114 carrying plasmid R1 or R46, either of which confers sulfamethoxazole resistance but not trimethoprim resistance (16), or plasmid R483 (11) or R751 (12), either of which confers resistance to trimethoprim but not sulfonamides, was also used.

Laboratory media. The laboratory media used are listed in the legend to Fig. 4.

Methods. Minimum inhibitory concentrations were determined as previously described (1). The fractional inhibitory concentrations were measured for each strain by determining the minimum inhibitory concentrations of both trimethoprim and sulfamethoxazole, each in the presence of sublimiting concentrations of the other end with the result expressed as a fraction of the MIC found in the absence of the other drug (9). The fractional inhibitory concentrations were plotted as an isobologram, and at the point of maximum potentiation the fractional inhibitory concentrations of both drugs were noted. The sum of these fractional inhibitory concentrations was taken as the fractional inhibitory index (6), and a value of less than 0.7 was taken as significant for showing synergy (13).

Susceptibility tests. Susceptibility tests were performed by culturing organisms overnight in Oxoid nutrient broth 2 (Oxoid, Basingstoke, United Kingdom). Unless otherwise stated, a 1/10,000 dilution was made in Davis-Mingioli minimal medium (8) and 1 ml of this dilution (ca. 10^4 to 10^5 organisms) was flooded onto the surface of Wellcotest Sensitivity Test Agar (Wellcome Research Laboratories, Beckenham, United Kingdom) in 8.5-cm-diameter plates containing 15 ml of medium. The excess liquid was pipetted off immediately, and the susceptibility disks were placed on the surface of the agar. The plates were incubated immediately at 37°C for 18 h. Organisms were classified as susceptible if the zone of inhibition around the disk was greater than 10 mm. Correlation analysis indicates that this cutoff point represents approximate minimum inhibitory concentrations of 10 mg of trimethoprim per liter and 20 mg of sulfamethoxazole

per liter, as determined on minimal medium (unpublished data). Synergy was identified by the bridging of zones of inhibition if the organisms were susceptible to both antimicrobial agents, a zone of inhibition between the disks if the organisms were resistant to both antimicrobial agents, or extension of a zone around the "susceptible" disk towards the "resistant" disk if the organisms were susceptible to only one of the antimicrobial agents (5, 17).

RESULTS

The minimum inhibitory concentrations of both trimethoprim and sulfamethoxazole were determined, and then each was measured again in the presence and absence of sublimiting concentrations of the other drug on Davis-Mingioli medium. The fractional inhibitory index was determined for each strain from this information. The results (Table 1) show the minimum inhibitory concentration determinations for representatives of the six main groups that are susceptible in vitro to the combination of trimethoprim and sulfamethoxazole. *E. coli* 114 was susceptible to both drugs, and the fractional inhibitory index indicated that synergy was prevalent. *E. coli* 114(R46) and *E. coli* 114(R1) were both sulfamethoxazole resistant and showed very similar resistance patterns. However, synergy could be demonstrated with *E. coli* 114 (R1). Similarly, although *E. coli* 114(R483) and *E. coli* 114(R751) showed identical resistance patterns, only with the latter could synergy be shown. *E. coli* R was resistant to both drugs, but synergy could be demonstrated.

When the six groups were tested for susceptibility to a single disk of 1 μg of trimethoprim and 50 μg of sulfamethoxazole, each showed a zone of inhibition around the disk greater than 10 mm in diameter. When the two drugs were tested in separate disks at the same concentrations, each group gave a different response to the two drugs (Fig. 1). The synergy between the two drugs could easily be seen with *E. coli* 114 (Fig. 1, 1), with bridging of the two inhibition zones (5, 17). The synergy between the drugs was visible with *E. coli* 114(R1) (Fig. 1, 3) and *E. coli* 114(R751) (Fig. 1, 5), by an extension of the zone around the "susceptible" disk towards the "resistant" disk in each case. With *E. coli* R, a zone of inhibition between the disks indicated synergy even though the strain was resistant to the individual disks. The demonstration of bacterial susceptibility and the synergy between the drugs was affected by a number of parameters.

TABLE 1. *Minimum inhibitory concentrations and fractional inhibitory indices of test organisms*

E. coli strain	Minimum inhibitory concn (μg/ml) of:		Fractional inhibitory index	Synergy
	Trimethoprim	Sulfamethoxazole		
114	0.4	0.5	0.5	+
114(R46)	0.2	4,000	1.0	−
114(R1)	0.4	8,000	0.2	+
114(R483)	2,000	0.5	1.2	−
114(R751)	2,000	0.5	0.5	+
R	16	1,000	0.4	+

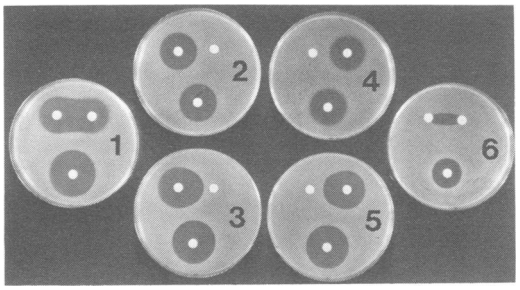

FIG. 1. *Bacterial susceptibilities of E. coli 114 (1), E. coli 114(R46) (2), E. coli 114(R1) (3), E. coli 114(R483) (4), E. coli 114(R751) (5), and E. coli R (6) to trimethoprim-sulfamethoxazole. Dilutions of 1/10,000 of overnight nutrient broth cultures were flood-seeded onto Wellcotest Sensitivity Test Agar plates. The trimethoprim disk (1 μg), on the left of each pair, and the sulfamethoxazole disk (50 μg), on the right, were placed 25 mm apart. The bottom disk on each plate contained both trimethoprim (1 μg) and sulfamethoxazole (50 μg).*

Content of the sulfamethoxazole disk. The optimum ratio for synergy with the most organisms is 1 part trimethoprim to 20 parts sulfamethoxazole (5). This is an absolute ratio related to the minimum inhibitory concentrations of both drugs. However, most complex laboratory media antagonize the action of sulfonamides to a greater extent than they do that of trimethoprim (1, 2) and therefore reduce the effective sulfamethoxazole concentration (2). Therefore, susceptibility tests were performed with single disks containing 1 μg of trimethoprim in conjunction with others containing increasing concentrations of sulfamethoxazole.

The results for *E. coli* 114 and *E. coli* 114(R1) (Fig. 2) showed that synergy could be demonstrated with susceptible *E. coli* 114 when the sulfamethoxazole disk contained 20 μg of the drug. However, synergy was less obvious when a disk of this content was used with sulfamethoxazole-resistant *E. coli* 114(R1). In either case, increasing the content of the sulfamethoxazole disk improved the demonstration of synergy. A disk of 50 μg of sulfamethoxazole showed synergy with both *E. coli* 114 and *E. coli* 114(R1). However, neither a disk of this sulfa-

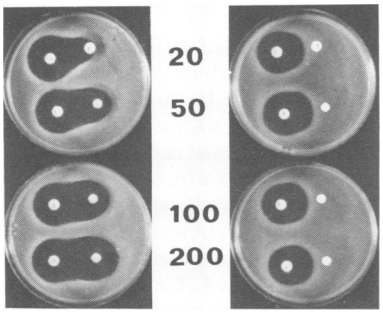

FIG. 2. *Effect of increasing sulfamethoxazole content on the demonstration of susceptibility and synergy. Dilutions of 1/10,000 of overnight broth cultures were flood-seeded onto Wellcotest Sensitivity Test Agar plates. Trimethoprim disks (1 μg), on the left of each pair, and sulfamethoxazole disks (contents shown in micrograms), were placed 25 mm apart. E. coli 114 is shown in the left column, and E. coli 114(R1) is on the right.*

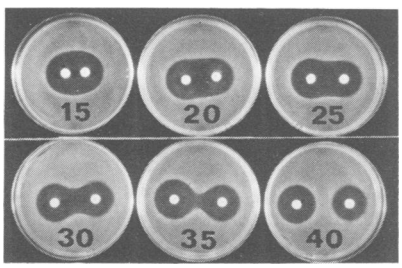

FIG. 3. *Effect of distance between disks on the demonstration of synergy. A 1/10,000 dilution of an overnight broth culture of E. coli 114 was flood-seeded onto Wellcotest Sensitivity Test Agar plates. A trimethoprim disk (1 μg), on the left of each pair, and a sulfamethoxazole disk (50 μg) were placed on the surface of the agar with their centers separated by various distances, shown in millimeters.*

methoxazole content nor one containing 100 or 200 μg of this drug gave any alteration in the shape of the zone around the trimethoprim disk with *E. coli* 114(R46) (data not shown). A disk content of 50 μg of sulfamethoxazole discriminated between the sulfamethoxaxole-resistant strains which showed synergy; in addition, the suitable laboratory media antagonize sulfamethoxazole by approximately 2.5 times (2), thus giving an effective trimethoprim/sulfamethoxazole ratio of about 1:20.

Distance between disks. The susceptibility tests were repeated with separate disks of 1 μg of trimethoprim and 50 μg of sulfamethoxazole with their centers between 15 and 40 mm apart. The results (Fig. 3) showed that with susceptible *E. coli* 114, the spacing of the disks was crucial. At 15 or 20 mm apart, the disks were too close together and the susceptibilities around the two disks overlapped each other. The bridging of the inhibition zones and the concomitant demonstration of synergy was found with a spacing of the disks at 25 or 30 mm. When this experiment was repeated with sulfamethoxazole-resistant *E. coli* 114(R1), 30 mm was too far apart for the disk positions, whereas 20 mm was too close. The same was true for *E. coli* 114(R751) and *E. coli* R. No matter what spacing was used, no demonstration of synergy was found with *E. coli* 114(R46) and *E. coli* 114(R483), so a distance between the disks of 25 mm was the optimum.

Choice of laboratory media. The choice of suitable laboratory media for the susceptibility testing of both sulfonamides and trimethoprim is most important (4, 14). Each strain was treated against one disk of trimethoprim (1 μg) and one of sulfamethoxazole (50 μg), spaced at 25 mm, on nine different laboratory media.

The results with susceptible *E. coli* 114 (Fig. 4) showed that, for the most part, the medium did not affect the demonstration of synergy as much as it affected the susceptibilities to the individual drugs. The only medium that abolished the demonstration of synergy was Oxoid blood agar base. Four media readily allowed the demonstration of synergy: Wellcotest Sensitivity Test Agar, Oxoid Diagnostic Sensitivity Test

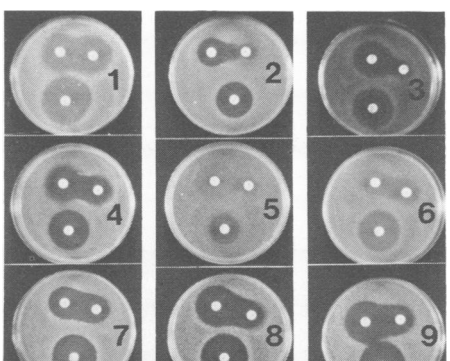

FIG. 4. *Effect of laboratory media on demonstration of synergy. A 1/10,000 dilution of an overnight broth culture of E. coli 114 was flood-seeded onto the surfaces of the following media: Davis-Mingioli minimal medium (1), Oxoid Mueller-Hinton agar (2), Oxoid MacConkey agar (3), Oxoid Diagnostic Sensitivity Test Agar (4), Oxoid blood agar base (5), Oxoid tryptone-soya agar (6), Difco Mueller-Hinton agar (7), Wellcotest Sensitivity Test Agar (8), and Oxoid Isosensitest Agar (9). A trimethoprim disk (1 μg), on the top left, and a sulfamethoxazole disk (50 μg), on the top right, were placed on the surface of the agar with their centers 25 mm apart. The bottom disk contained both trimethoprim (1 μg) and sulfamethoxazole (50 μg).*

Agar, Oxoid Isosensitest Agar, and Difco Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). These media also show the least antagonism to the actions of trimethoprim and sulfonamides (2, 3). When the other strains were tested in the same manner, the four media listed above invariably gave the best demonstration of synergy as well as drug susceptibility.

When this experiment was repeated with laboratory media containing 4% lysed horse blood, there was no considerable improvement in the demonstration of synergy.

Inoculum of bacteria. The susceptibility of bacteria to both trimethoprim and sulfamethoxazole is dependent on the inoculum used (4). An overnight broth culture of *E. coli* 114 was diluted in 10-fold steps, and each dilution was used as an inoculum to flood the surface of a Wellcotest Sensitivity Test Agar plate. A 1-μg trimethoprim disk and a 50-μg sulfamethoxazole disk were placed 25 mm apart on the agar surface. At a dilution of less than 1/100 there was no demonstration of synergy or sulfamethoxazole susceptibility, even with the washed culture that had been centrifuged and resuspended in Davis-Mingioli medium (Fig. 5). As the dilution was increased, synergy was more readily observable. A dilution of 1/10,000 (between 10^4 and 10^5 organisms per ml) was found to be optimum, and there was no improvement in the demonstration of synergy or sulfamethoxazole susceptibility if the dilution was greater. A 1/10,000 dilution was also found to be the most suitable for resistant organisms.

Susceptibilities of clinical isolates. The susceptibilities of a random collection of 101 clinical enterobacteria isolated from significant bacteriuria specimens submitted to the bacteriology laboratories of the Royal Infirmary, Edinburgh, United Kingdom, were examined. Each isolate was found to be susceptible to the combined susceptibility disk containing 1 μg of trimethoprim plus 20 μg of sulfamethoxazole. However, these isolates were retested by flooding a Wellcotest Sensitivity Test Agar plate with a 1/10,000 dilution of an overnight broth culture. A 1-μg trimethoprim disk and a 50-μg sulfamethoxazole disk were placed 25 mm apart. The results (Table 2) showed the relative proportions of isolates tested, 48 were susceptible to both drugs. The resistance pattern was invariably concomitant with the demonstration of synergy. Of the 39 isolates that were resistant to sulfamethoxazole, 20 were also susceptible to trimethoprim in the absence of demonstrable synergy and 15 had the same resistance pattern with synergy. The four remaining isolates were resistant to both drugs, and synergy was demonstrated. Of the 21 trimethoprim-resistant, sulfamethoxazole-susceptible isolates, only in 1 could synergy not be shown.

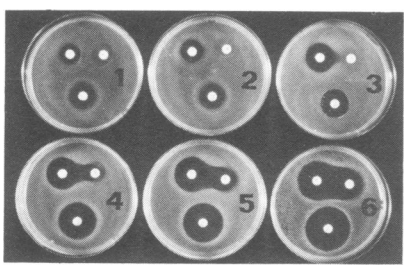

FIG. 5. *Effect of inoculum on the demonstration of synergy and bacterial susceptibility. An overnight broth culture of E. coli 114 was diluted as shown. A trimethoprim disk (1 μg), on the top left, and the sulfamethoxazole disk (50 μg), on the top right, was placed on each plate. The bottom disk contained both trimethoprim (1 μg) and sulfamethoxazole (50 μg). (1) Overnight broth culture; (2) washed broth culture resuspended in minimal medium; (3) 1/10^1 dilution in minimal medium; (4) 1/10^2 dilution; (5) 1/10^3 dilution; (6) 1/10^4 dilution.*

DISCUSSION

The majority of diagnostic laboratories test bacterial susceptibility to the components of cotrimoxazole with a single susceptibility disk containing both trimethoprim and sulfamethoxazole. This may often give an erroneous account of the true susceptibility pattern of the organisms being tested. This paper describes a method aimed at improving the information obtained from the susceptibility test in order to provide the clinician the best opportunity to determine subsequent therapy. In the United Kingdom, as in some other countries, trimethoprim is now being marketed on its own, as well as in combination with sulfamethoxazole. This

TABLE 2. *Analysis of 101 urinary isolates that were classified as susceptible to a combined trimethoprim-sulfamethoxazole disk, giving a zone of clearing around the disk of greater than 10 mm*

Characteristics[a]	No. of isolates
Tp^s Su^s with synergy	48
Tp^s Su^r with synergy	15
Tp^r Su^s with synergy	13
Tp^r Su^r with synergy	4
Tp^s Su^r without synergy	20
Tp^r Su^s without synergy	1

[a] Isolates characterized as susceptible to trimethoprim (Tp^s) or sulfamethoxazole (Su^s) gave zones of clearing greater than 10 mm; isolates characterized as resistant to trimethoprim (Tp^r) or sulfamethoxazole (Su^r) gave no zones.

method for susceptibility testing facilitates the decision whether to use the combination or trimethoprim.

About 20% of bacteria isolated from urinary tract infections in Edinburgh are resistant to sulfamethoxazole, and there is no synergy that can be demonstrated (S. G. B. Amyes and W. A. Telfer Brunton, unpublished data). Treatment of these bacteria with the combination of the two drugs is, effectively, treatment with trimethoprim alone. The majority of the side effects of the combination arise from the sulfonamide component (15), and, in the case of these resistant bacteria, therapy with trimethoprim alone would be preferable to administration of a drug that may do more harm than good. In susceptible bacteria and in some that are resistant to one component, synergy may be important. In these cases administration of combined therapy, based on a full susceptibility test result, may be used to deal with the infection.

One group of bacteria is resistant to both drugs individually but appears sensitive to the combined disk because of the synergy between the drugs. In this case, the clinician should be certain that the optimum ratio for synergy will be present at the site of action before prescribing combined therapy.

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