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TECHNICAL METHOD

A DIRECT DISC TECHNIQUE FOR DETERMINING THE SENSITIVITY OF ORGANISMS TO THE SULPHONAMIDES

BY

R. W. FAIRBROTHER AND AHALYA RAO

From the Department of Clinical Pathology, Manchester Royal Infirmary

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The laboratory control of chemotherapy is becoming increasingly important for two reasons: (1) the majority of common infections may be caused by a wide variety of organisms, the nature of which cannot be determined by clinical methods; and (2) members of individual bacterial species or groups are tending to vary considerably in their reactions to the different agents. It is, however, necessary that laboratory tests should be relatively simple and give rapid and reliable information if they are to provide a useful control for routine purposes.

In the case of the antibiotics, these criteria have been largely fulfilled and laboratory control of therapy is widely used. With the sulphonamides, however, the practice is still restricted, due mainly to technical difficulties.

Laboratory tests with sulphonamides are greatly influenced by many factors, in particular the composition of the medium, the concentration of the organisms, and the period of incubation. The significance of the tests may also be uncertain, as marked discrepancies between *in vitro* and *in vivo* results do occur. Nevertheless sensitivity tests can offer useful information provided the conditions of the test are carefully standardized.

Several methods are available, e.g., the serial dilution technique and various forms of agar-diffusion, such as the ditch-plate, streak-plate, whole-plate, and the use of discs. The disc technique, at present used for the sulphonamides in some laboratories, is different from that used for the antibiotics; it is a two-stage method in which the discs are placed on the medium for a fixed period, after which they are removed and the plates seeded with the organism to be tested (Evans, 1948; MacFarlane, 1951).

The antibiotic disc test is a simple one-stage technique and, as it has given excellent results in routine practice, it was decided to investigate the possibility of adapting this to the testing of the sulphonamides.

Dried discs and tablets were tried and the following sulphonamides examined:—Sulphathiazole, sulphadimidine ("sulphamezathine" by Imperial Chemical (Pharmaceuticals), sulphafurazole ("gantrisin" by Roche) and a sulphasomidine ("elkosin" by Ciba).

Preparation of Materials

Medium.—It is essential in comparative work with the sulphonamides that all media should be standardized and that any sulphonamide-antagonizers should be neutralized. In consequence, lysed horse-blood agar was used throughout the investigation (Harper and Cawston, 1945; Walker, Philip, Smyth, and McLeod, 1947). The supernatant plasma of oxalated horse-blood was replaced by sterile distilled water and the reconstituted cell suspension well shaken; lysis was completed by alternate freezing and thawing, and the solution was stored in the refrigerator. The final medium was prepared by the addition of the solution to melted nutrient agar to give a final concentration of 5%.

Sulphonamide Solutions.—The sulphonamides are relatively insoluble compounds but, in the case of sulphathiazole and sulphadimidine, suitable dilutions were readily prepared from the soluble intravenous products. Sulphafurazole and "elkosin" were only available in the crystalline or powder form and the required solutions in distilled water were effected by boiling. In all cases a stock solution (1 g. in 10 ml.) was prepared and stored at room temperature; some recrystallization occurred on cooling with sulphafurazole and "elkosin" but this deposit re-dissolved on boiling, and further dilutions were then made as quickly as possible.

Discs.—Discs of 8 mm. diameter were punched from blotting-paper (Ford Mill 428); a different colour was used for each sulphonamide. After sterilization in the hot-air oven, a standard drop (50-dropper) of the four different sulphonamide solutions (1 in 80 final dilution) was added to each disc, giving a total amount of 0.25 mg. per disc.

The discs were stored in sterile universal containers or Petri dishes. They maintained their potency for a considerable time; chemical assays were made at

intervals up to five months and the discs proved very stable. There was no deterioration.

Tablets.—The tablets were prepared by Messrs. Evans Medical Supplies Ltd. Original supplies proved unsatisfactory as they gave irregular results and were unsuitable for use in sensitivity tests. The difficulties of preparation were apparently overcome, as later preparations were subjected to rigorous trial and proved quite consistent, giving uniform results by biological and chemical tests.

Tablets containing 1, 2, and 10 mg. sulphonamide were tried, but it was found that the zones of inhibition given by the 10-mg. tablet were only slightly larger than those produced by the 1-mg. tablets. Chemical assays of the tablets before and after incubation indicated that while the 1-mg. tablets lost approximately 60% of the sulphonamide, only about 20% diffused from the 10-mg. tablets. It was decided to use the 1-mg. tablets for the tests.

The Inoculum (Bacterial Suspensions).—All organisms were recently isolated from infective processes. It is generally accepted that the concentration of the inoculum exercises a great influence on the final result and that the degree of dilution varies with different organisms. As a rule the organisms were seeded into broth and, after six hours' incubation at 37° C., tenfold dilutions (up to 1 in 10^8) were made in sterile distilled water. The more fastidious organisms, such as pneumococcus, *Strept. haemolyticus,* and meningococcus, were grown in special media, e.g., O'Meara and Brown (1936), and serum broth, for 24 hours before dilution.

Sensitivity Tests

Ditch-plate Method.—Ditches were cut in the lysed blood agar and filled with 5 ml. of similar media containing the various concentrations of sulphathiazole and sulphadimidine. In preliminary trials a wide range (0.6–10 mg.) was used, but eventually a sulphonamide strength of 1.25 mg. per 5-ml. ditch was adopted for the investigation.

The plates were left for six hours at 37° C. or overnight in the refrigerator before use in order to allow adequate diffusion of the sulphonamide from the ditch. They were then seeded by streaking across the ditch with a standard loop (2.5 mm. diameter). Ten or twelve streaks were made on each plate, and at least four dilutions of each organism were tested. A known sensitive organism was invariably used as a control. Readings were made after incubation at 37° C. for 18 hours; the dilution producing discrete colonies was that used for making the final assessment of sensitivity to the drug.

The most suitable dilutions for this technique varied with the different organisms; those mainly used in this investigation were 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}. High dilution was essential for most organisms, but it is interesting to note that this had little effect on the results given by *Staph. aureus.*

The "Whole-plate" Method.—Plates containing 15 ml. of the lysed blood-agar plus the sulphonamides were prepared. The sulphonamides used were sulphathiazole and sulphadimidine in concentrations of 25 and 12.5 mg. per 100 ml. The plates were stored in the refrigerator, usually overnight, and after thorough drying were seeded in prescribed areas with a standard drop of the tenfold dilutions of the bacterial suspension; a control plate, free from sulphonamide, was always prepared for each test, and a known sensitive strain was used as a control organism. After incubation at 37° C. for 18 hours readings were made. Important factors in the final assessment of sensitivity were the differences in growth on the control and test plates, particularly with the dilutions producing discrete colonies on the control plate.

The Dried Disc Method.—The technique was similar to that used for the examination of the antibiotics (Fairbrother and Martyn, 1951). Pour-plates with appropriate dilutions of the bacterial suspensions were prepared on the lysed blood medium; after the excess of culture had been pipetted off the discs were placed on the surface of the medium at convenient intervals and the plates incubated at 37° C. for 14 to 18 hours. Readings were then made; the degree of sensitivity was assessed by the zone of inhibition around the discs.

Chemical analysis of the discs, before and after incubation, showed that 90% of the sulphonamides had been lost, probably by diffusion into the medium during this period.

Many dilutions of the organisms were investigated during the preliminary trials, and it was found that, while high dilution was usually necessary, the optimal strength varied with the different organisms. The following dilutions tended to give satisfactory results and were adopted for the trial: *Staph. aureus,* coliforms, and Friedlander's bacillus = 1/2,000 (six-hour culture); *Proteus* = 1/20,000 (six-hour culture); *Ps. pyocyanea* = 1/100,000 (six-hour culture); *Pneumococcus* and *Strept. haemolyticus* = 1/500 (24-hour culture).

Tablet Method.—The technique was similar to that of the disc method, with tablets of sulphathiazole and sulphadimidine substituted for the discs.

Results

The investigation was divided into two parts: (1) A comparison of the four different agar diffusion techniques using sulphathiazole and sulphadimidine; (2) a trial by the dried disc technique of four potent sulphonamides: sulphathiazole, sulphadimidine, sulphafurazole, and "elkosin."

The first part of the investigation was made with 190 organisms freshly isolated from routine material of diverse origin. Each organism was tested simultaneously by the four methods under comparable conditions; the tablets and discs were added to the same pour-plate (Fig. 1). The results are given in Table I.

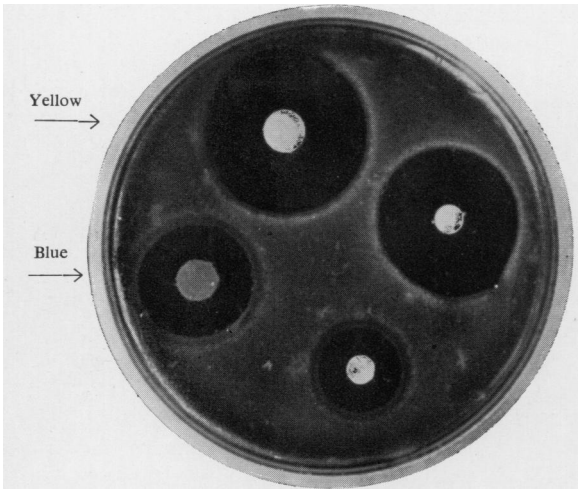

FIG. 1.—*Staph. aureus* tested against sulphathiazole (a yellow disc) and sulphadimidine (a blue disc) by the disc and tablet techniques. (Disc and tablet of the same drug are adjacent.)

TABLE I

SENSITIVITY TESTS ON 190 ORGANISMS BY FOUR DIFFERENT METHODS

Organism	Number Tested	Number Sensitive to Sulphathiazole				Number Sensitive to Sulphadimidine			
		Ditch Plate	Whole Plate	Disc*	Tablet*	Ditch Plate	Whole Plate	Disc	Tablet
Staph. aureus ..	75	52	52	52	52	48	48	48	48
Strept. haemolyticus ..	24	18	18	18	18	15	15	15	15
Strept. faecalis ..	11	0	0	0	0	0	0	0	0
Pneumococcus ..	10	10	10	10	10	10	10	10	10
N. meningitidis ..	4	4	4	4	4	4	4	4	4
Bact. coli	50	37	37	37	37	8	9	10	9
Ps. pyocyanea ..	16	9	9	9	9	0	0	0	0
Total ..	190	130	130	130	130	85	86	87	86

* In the disc and tablet techniques sensitivity is equivalent to a zone of inhibition at least 15 mm. in diameter.

The results of the four different methods were remarkably consistent. With sulphathiazole there was complete agreement, while in the case of sulphadimidine a very slight difference was observed only with the coliform group. Sulphathiazole was more active than sulphadimidine, particularly against the Gram-negative bacilli. It is interesting to note the irregular behaviour of individual members of some species or groups to both sulphonamides; only strains of the pneumococcus, meningococcus and *Strept. faecalis* gave completely consistent results. It follows that even though the identity of an organism has been established, this may not indicate the reaction to the sulphonamides of the particular strain under investigation.

A further series of 120 organisms was tested against four active sulphonamides by the dried disc technique (Table II). The discs were placed on the same pour-plate of the organism and thus the conditions of the test were strictly comparable (Fig. 2).

Readings were obtained without difficulty and, except in the case of *Ps. pyocyanea*, the

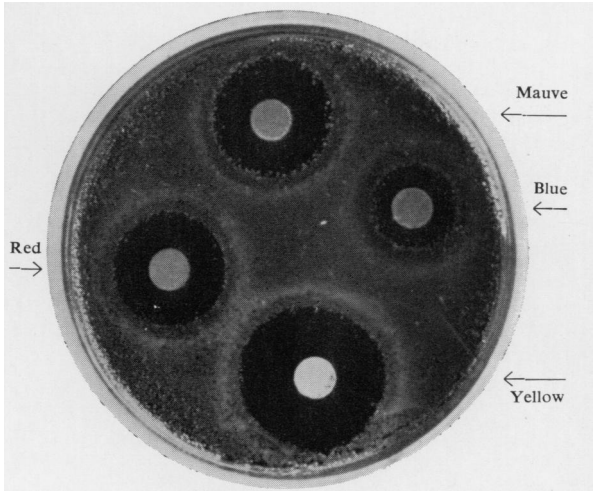

FIG. 2.—*Bact. coli* tested by the disc technique against sulphathiazole (a yellow disc), sulphafurazole (a mauve disc), "elkosin" (a red disc) and sulphadimidine (a blue disc).

TABLE II

RESULTS BY THE DRIED DISC TECHNIQUE

Organism	Number Tested	Result	Sulphathiazole		Sulphadimidine		Gantrisin		Elkosin	
			No. Sensitive	Total (%)	No. Sensitive	Total (%)	No. Sensitive	Total (%)	No. Sensitive	Total (%)
Staph. aureus ..	35	++	25	80	15	77	24	80	20	74
		+	3		12		4		6	
Strept. haemolyticus (Group A)	10	++	0	60	0	40	0	60	0	40
		+	6		4		6		4	
Pneumococcus ..	4	++	4	100	4	100	4	100	4	100
		+	0		0		0		0	
Coliforms ..	43	++	33	86	4	84	28	84	31	86
		+	4		32		8		6	
Ps. pyocyanea ..	12	++	6	67	0	0	0	17	0	25
		+	2		0		2		3	
Proteus	16	++	14	94	9	88	12	88	12	88
		+	1		5		2		2	
Total ..	120									

++ indicates that the zone of inhibition exceeded 25 mm. in diameter and + that it equalled 10 to 25 mm. in diameter.

sulphonamides gave similar qualitative results. Sulphathiazole was the most potent agent by these tests, but all compounds exhibited a wide range of activity. The quantitative differences in the zones of inhibition are of doubtful value in assessing therapeutic activity, as the *in vivo* action of the sulphonamides is influenced by many factors which cannot be controlled in the *in vitro* tests.

Discussion

These results indicate that the dried disc technique provides a useful routine method for determining the sensitivity of organisms to the sulphonamides. The technique is relatively simple and allows several sulphonamides to be tested simultaneously on one plate; a further advantage is that the technique is similar to that frequently used for the routine testing of the antibiotics. It is, however, a laboratory test and merely gives an indication of the activity of the sulphonamides under strictly *in vitro* conditions. It is well recognized that the clinical response does not always run parallel with the laboratory tests and, in the selection of a sulphonamide for clinical purposes, such factors as solubility, absorption, excretion, toxicity, and degree of acetylation and binding to the plasma proteins, must also be considered. The sulphonamides are primarily bacteriostatic agents and, in therapy, it is essential that they should reach the focus of infection in an active form and that an effective level should be maintained for some time.

Nevertheless, while sensitivity tests may provide limited information about the suitability of a sulphonamide for clinical purposes, they give a useful indication of the potential activity of the agent.

Summary

A one-stage dried disc technique for determining sensitivity to the sulphonamides has been described. This has given results comparable with those of other agar-diffusion methods.

We wish to thank Messrs. Evans Medical Supplies Ltd. for providing the sulphonamide tablets.

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