Use of sensitivity discs as primary antibiotic standards in MIC determination

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SUMMARY A method for the determination of minimum inhibitory concentrations (MICs) is described, in which commercially prepared antibiotic sensitivity testing discs are used as primary antibiotic standards from which the necessary dilutions are prepared in WHO Perspex haemagglutination plates. The materials required are readily available in most laboratories at a cost in time and reagents that compares favourably with most traditional methods of MIC testing. The same techniques may also be used to demonstrate combined antibiotic activity and to differentiate between bacteriostatic and bactericidal activity, with little expenditure of laboratory resources.

Most laboratories in this country use a disc diffusion method to test bacteria for susceptibility to antibiotics; results are reported as sensitive or resistant with an intermediate category of moderately sensitive. An international study (Ericsson and Sherris, 1971) suggested that routine sensitivity testing methods should be based on the relation between the minimum inhibitory concentration (MIC) and levels of antibiotic found in body fluids. Estimation of the MIC is necessary for isolates from endocarditis and from serious infections such as septicaemia, and for organisms giving an equivocal result in a disc test. Automated methods have been developed for determining MICs based on photometric monitoring of bacterial growth (Autobac, Pfizer, Diagnostic Division and MS-2, Abbott Laboratories) or automation of titrations in microtitre plates (MIC 2000, Dynatech). A microtitre plate containing freeze-dried dilutions of four different antibiotics is available, being designed to measure the MIC of a test organism and a control, and is suitable for performing MICs in clinical laboratories (Sensititre, Seward Laboratories). This communication describes the use of commercially prepared antibiotic sensitivity testing discs as the source of antibiotic standard for performing MICs.

Material and methods

Oxoid brand sensitivity discs and Multodisks taken from batches in routine use in the laboratory were

used throughout these investigations. The growth medium used was Iso-Sensitest broth (Oxoid CM 473).

In the tests reported the organism used was *Staphylococcus aureus* NCTC 6571. Testing was carried out in WHO Perspex haemagglutination plates. Before use these were disinfected by immersion in dilute hypochlorite solution (100 ppm available chlorine) for 30 minutes, after which the excess moisture was shaken off and the plates were dried face down on paper hand towels in the incubator. Plates were then stored with lids on until required; if lids are not available the sterile plastic bags in which 9 cm plastic petri dishes are purchased make a suitable alternative. As a further precaution against contamination the plates were placed under an ultraviolet lamp for three minutes just before use, although subsequent studies have shown no increased rate of contamination when this step is omitted.

For dispensing broth and making doubling dilutions, automatic pipettes (Excel) with 1000 μ l and 200 μ l tips were used. New tips were used without sterilisation although they were autoclaved if re-used.

The rate of elution of antibiotic from sensitivity discs and the tips of Multodisks was examined by immersing them in 1000 μ l volumes of broth for varying periods of time up to 20 minutes, and then using the broth to perform MICs to the standard organism. In the case of Multodisks, the necks that remained were removed and placed in broth, which was checked for evidence of antibiotic activity.

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Figure A WHO plate in which eight penicillin discs have been used to prepare MICs to the same staphylococcus. Six results are within the mode.

DISC METHOD

Ten drops of a 1/100 dilution of a 24-hour culture of the test organism were added to 10 ml of broth, giving a final bacterial concentration of approximately 10^5 organisms per ml.

One thousand microlitres of broth was placed in the first well of a WHO plate and 200 μ l of broth was placed in subsequent wells of the same row (Figure). A sensitivity disc was placed in the first well, and, after allowing a period of one minute for elution of the antibiotic from the paper, doubling dilutions were made along the row by serially transferring 200 μ l volumes. In order to ensure adequate mixing, the contents of the tip were withdrawn and expelled three times in each well before proceeding to the next. The plate was covered, placed in a plastic bag to prevent evaporation, and incubated overnight at 37°C. The wells were examined for turbidity or the formation of a button of bacterial cells. Results were recorded as no growth (–), faint growth (±), or growth (+). It was found convenient to leave the disc in the first well to indicate the antibiotic used and its concentration, while a double volume left in the last well, rather than the usual practice of discarding one volume, shows that the dilution series has been made.

Tube MICs were performed in 75 × 12 mm glass tubes using 1000 μ l volumes of broth inoculated as for the disc method. Antibiotic standards were prepared from pharmaceutical preparations of benzylpenicillin (Crystapen; Glaxo) and gentamicin (Genticin; Nicholas).

Plate MICs were carried out by using 200 μ l volumes of the reagents used for tube MICs, dispensed in WHO plates as used in the disc test.

Results

The activity of Multodisks was shown to be located entirely in the tips, and excision through the neck did not result in loss of antibiotic. Studies in which sensitivity discs and Multodisk tips were immersed in broth for periods up to 20 minutes showed that elution of antibiotic took less than one minute, any gain in potency after this period being too small for detection by methods using doubling dilutions. It was found that the working time required to set up an MIC by the disc method was less than five minutes.

The results obtained by performing MICs to penicillin in tubes and plates are shown in Table 1. Section A shows the results of 10 tube MICs using a pharmaceutical preparation of penicillin when 70% of the results are seen to be modal. In section B, the same reagents have been used to perform 50 MICs in plastic trays, and 82% of results fall within the same modal concentration.

In section C, the same organism is tested using 100 penicillin discs from five containers bearing the same batch number, by the new method; the modal concentration is again the same and 77% of results are modal. In all cases the results are within one dilution step of the mode which confirms the validity of the titration methods and the use of this batch of discs as primary penicillin standards.

Table 2 shows the results obtained when the staphylococcus was tested in 60 MICs, in which a pharmaceutical preparation of gentamicin was used, and a further 300 tests in which separate 10 μ g gentamicin discs provided the source of the antibiotic. In sections A and B the modal result is seen to be the same whether the test is carried out in tubes or in plates. In section C, 100 tests have been made from gentamicin discs and, while the modal concentration is the same, only 51% of results are modal although all results are within one dilution of the mode. In section D, the test has been modified by eluting the same batch of discs in a larger volume of broth (8 ml) in order to reduce the number of dilution steps necessary. Although the reagents used in sections C and D are the same, the number of results which fall within the modal concentration when the latter modification is used is now seen to be 89%. These results emphasise the importance of avoiding as many dilution steps as possible when using doubling dilution techniques, a fact which is well recognised by the experienced serologist.

Discussion

The principal aim of this paper is to promote consideration of commercially prepared sensitivity testing discs as primary antibiotic standards. There are at the present time no legally enforceable standards governing the antibiotic content of such

Table 1 Results of 160 benzylpenicillin MICs carried out by three different methods

		Benzylpenicillin units/ml								Titrations	
		1.5	0.75	0.4	0.2	0.1	0.05	0.025	0.0125	No.	Percent
A	Tube MICs	—	—	—	—	—	—	±	+	7	70
	1000 µl volumes	—	—	—	—	—	—	+	+	3	30
										10	100
B	Plate MICs	—	—	—	—	—	—	±	+	41	82
	200 µl volumes	—	—	—	—	—	—	+	+	9	18
										50	100
C	Disc method	—	—	—	—	—	—	—	+	12	12
	200 µl volumes	—	—	—	—	—	—	±	+	77	77
		—	—	—	—	—	—	+	+	9	9
		—	—	—	—	—	±	+	+	2	2
										100	100

+ growth; ± faint growth; — no growth

Sections A and B are based on a pharmaceutical preparation of penicillin while section C is based on discs.

Table 2 Results of 360 gentamicin MICs carried out by four different methods

		Gentamicin concentration (μg/ml)								Titrations	
		10	5	2.5	1.25	0.6	0.3	0.15	0.08	No.	Percent
A	Tube MICs	—	—	—	—	—	—	±	+	2	20
	1000 μl volumes	—	—	—	—	—	—	+	+	8	80
										10	100
B	Plate MICs	—	—	—	—	—	—	—	+	2	4
	200 μl volumes	—	—	—	—	—	—	±	+	9	18
		—	—	—	—	—	—	+	+	32	64
		—	—	—	—	—	±	+	+	7	14
										50	100
C	Disc method	—	—	—	—	—	—	—	+	12	12
	200 μl volumes	—	—	—	—	—	—	±	+	7	7
		—	—	—	—	—	—	+	+	51	51
		—	—	—	—	—	±	+	+	25	25
		—	—	—	—	—	+	+	+	5	5
D	Modified disc method				—	—	—	±	+	10	5
	200 μl volumes				—	—	—	+	+	178	89
					—	—	±	+	+	12	6
										200	100

+ growth; ± faint growth; — no growth

Sections A and B are based upon a pharmaceutical preparation of gentamicin while sections C and D are based on discs.

discs in the UK, but the major manufacturers claim to meet the FDA (USA) standards, which require discs to contain 67-150% of the stated antibiotic content. The new DIN (West Germany) standards specify 90-120% of the stated content and, with the improvements which have taken place in modern disc manufacture, at least one commercial source in this country feels that these standards can be met. The DIN standards are in fact very similar to the legally enforceable standards applicable to pharmaceutical antibiotic preparations in this country, and many laboratory workers use such preparations as standards without consideration of the inherent packaging inaccuracies. It therefore seems reason-

able to use a disc as an antibiotic standard in tests in which twofold errors in end-point are considered acceptable. Recently, antibiotic impregnated sheets of paper to fit the base of a 9 cm petri dish and designed to elute a range of antibiotic concentrations into agar (Mast Laboratories) have been introduced to provide a source of standards for performing MICs by the plate test technique. Such a method indicates the feasibility of using antibiotic standards dried on to paper. Discs used for such a purpose, as for routine tests, must be carefully stored and used under the conditions recommended by the manufacturer, in order to prevent loss of potency. Where a choice of antibiotic content is available, those of

high content are likely to suffer less loss of potency given the same conditions (Brown and Kothari, 1975), although this advantage may be offset by the larger number of dilution steps required, unless, as is seen in Table 2, section D, the disc is first eluted in a larger volume of broth. The best results are likely to be obtained when discs of high content are eluted in a volume of broth that is large enough to ensure that the subsequent number of dilution steps required to reach the MIC is small; this volume will vary with the MIC of the organism being tested but can usually be anticipated from the zone size obtained in diffusion tests which have been carried out as part of the laboratory routine.

Other media can be used with this technique, and both Mueller Hinton broth (Oxoid CM 405) and indicator serum broth (Stokes, 1968) have been found satisfactory, although the latter is unsuitable for sulphonamides and trimethoprim. When testing clinical isolates the medium used and inoculum size may well affect the result obtained, particularly when gentamicin is being used. Such variation is acceptable only if controls of known MIC are titrated at the same time; this is best done by elution of the disc in sterile broth to give a standard solution from which both test and control are titrated in pre-seeded broth.

It has been found that, with practice, the working time required to set up an MIC by the new method is less than five minutes, while one eight-tipped Multodisk can be used to provide several MICs in 15 minutes working time and requires only 25 ml of suitable broth. The disc manufacturers state that some cephalosporins may not show complete elution in one minute although the residual amount after this time appears to be very small.

Reproducibility of results has been shown to be comparable to those obtained by tube MICs. A small number of tests with the Sensititre method also gave comparable results. No extensive comparative trial with the Sensititre has been made but the manufacturers of this method claim that staphylococcus MICs for all antibiotics in their range show 68.1% of results to be modal. By contrast, the disc

method shows 77% modality for penicillin and 89% modality for gentamicin MICs.

The use of discs lends itself to tests of combined antibiotic activity, as described by Garrod *et al.* (1973), in which antibiotics are used in various combinations at concentrations of 10 µg/ml. These tests can be very time-consuming if fresh standards have to be prepared from pharmaceutical preparations, but can easily be carried out by placing two discs of 10 µg content in 1 ml of pre-seeded broth, and a small number of such tests have been performed. Since in these tests it is necessary to mix the contents adequately before subcultures are made to differentiate between bactericidal and bacteriostatic activity, it was found to be more convenient to perform them in test tubes rather than in wells which are difficult to mix when they contain this relatively large volume of broth. Using this technique, it was easy to differentiate between bacteriostatic and bactericidal combinations.

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