Light-scattering methods for antibiotic sensitivity tests

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SUMMARY The total intensity of light scattered in the angular range 30°-130° by a suspension of bacteria can be used as the basis of a method of antibiotic sensitivity testing. A procedure is given in which the bacteria are incubated in the presence of antibiotic, as a test, and in its absence, as a control, for 60 minutes. An incubation time of 90 minutes gives slightly more reliable results. With this increased incubation time it is necessary to measure the intensity of scattered light only at a single fixed angle or, at most, a few angles. Measuring the angular dependence of the scattered intensity does not allow the incubation period to be reduced.

It has been proposed that the angular dependence of the light scattered by a suspension of bacteria could be used as the basis of a rapid method of antibiotic sensitivity testing.¹ The Differential I light-scattering photometer (Science Spectrum Inc, Santa Barbara, California, USA) was developed for this purpose.² It can be used to plot the logarithm of the intensity of the scattered light against the scattering angle over some required angular range. The result has been called a differential light-scattering (DLS) curve.

The principles involved in sensitivity testing are as follows. Two samples, of identical volume, are taken from a homogeneous suspension of bacteria. Both samples are cultured in a liquid growth medium. Antibiotic is added to the test sample but not to the control sample. When a suitable incubation time has elapsed, DLS curves are obtained from both. A significant difference between the curves indicates that the antibiotic affects the growth or structure of the bacteria.

In principle, the curves can differ in two respects. A difference in scale indicates a difference in the total number of bacteria in each sample—the greater the intensity, the higher the number. Any difference in shape between the curves indicates a change in bacterial morphology. The problem which then arises is to decide whether the difference in scale or shape is significant.

The displacement index, *D*, has been defined by

$$D = \text{Area between curves} / \text{Angular range}$$

and allows for differences in both scale and shape.³ Differences in shape alone can be allowed for by

calculating the area between the two curves and dividing by the angular range, as before, after they have been scaled together so as to have the same average values over their angular range. The result is called the morphological index, *M*, and is combined with *D* to give a total score, *S*, defined by

$$S = D + 3M$$

for the sensitivity tests.³ (The factor of 3 was chosen to obtain equal contributions from the *D* and 3*M* terms after the bacteria had been incubated for 90 minutes.³)

The intensity of the light scattered at a single fixed angle had already been used for sensitivity testing.⁴ But this technique could yield incorrect results if the effect of the antibiotic were greatly to change the shape of the DLS curve.⁵ This effect will be considered in greater detail later (see Results).

Our experiments were designed to investigate these various methods of antibiotic sensitivity testing using the Differential I photometer. We find that the morphological term, *M*, is of little value as the total score, *S*, provides no more significant information than the displacement index, *D*. Furthermore, measurements at a fixed angle are not often misleading. The occasional problems that arise could be overcome with a simpler instrument than the Differential I. We also find that a suitable incubation time is 60 or 90 minutes, depending on the exact method chosen, and that the results are not significantly influenced by noise in the DLS curves.

In these experiments the light-scattering results were compared with the results of agar plate sensitivity tests. Preliminary experiments showed that the light-scattering indices, *D* and *S*, were not strongly correlated with the zone diameters in the

plate tests. Therefore these tests were used to decide whether bacteria were sensitive or insensitive to a given antibiotic. It was necessary to find a boundary level for the value of each index above which the bacteria would be judged to be sensitive to the antibiotic in the light-scattering tests. The number of times when agar plate and light-scattering tests disagreed was plotted against trial boundary levels; a minimum in the resulting graph indicated the optimum level. This procedure was repeated for D, S, and the results obtained from single angle measurements after three different incubation periods. The entire set of analyses was repeated with cubic spline representations of the curves, which correspond to noise-free DLS results,⁶ in order to assess the effects of noise on the results.

Material and methods

BACTERIA AND ANTIBIOTICS

Two hundred samples of bacteria were used in the tests. These were chosen as representative of a wide range of clinical samples obtained for routine testing.

Antibiotics were used in the form of impregnated paper discs (Mast Laboratories Ltd, Liverpool, UK) and were stored at 4°C. The antibiotics tested for use against the different genera of bacteria are listed in Table 1.

Table 1 Antibiotics used against different types of bacteria

Bacteria	Antibiotic
Gram-positive	
Staphylococcus	Penicillin, methicillin, erythromycin, lincomycin, fucin
Streptococcus	Penicillin, erythromycin, lincomycin, tetracycline
Gram-negative	
Klebsiella and Escherichia	Ampicillin, tetracycline, mecillinam, streptomycin, gentamicin
Proteus	Ampicillin, carbenicillin, streptomycin, gentamicin
Pseudomonas	Streptomycin, carbenicillin, gentamicin

AGAR PLATE TESTS

Plate sensitivity tests were carried out by a method based on those described previously.^{7,8} Standard organisms were strains of *Staphylococcus aureus* (when testing Gram-positive spheres), *Escherichia coli* (when testing Enterobacteriaceae), and *Pseudomonas pyocyanea* (when testing the genus *Pseudomonas*). Problems encountered in this technique are discussed by Stokes and Waterworth.⁸

Zone diameters were measured, to the nearest millimetre, using callipers. Bacteria were classified as sensitive if the zone diameter exceeded 18 mm.

LIGHT-SCATTERING TESTS

All incubations referred to below were at 37°C, and when a medium was prewarmed it was always to this same temperature. Bacteria were first incubated, usually overnight, on blood agar plates. A few colonies were then transferred to a small bottle containing prewarmed brain-heart infusion (4 ml; full strength), using a flamed wire loop, and incubated for 30 minutes. After incubation the bottle was shaken, to ensure an even distribution of bacteria, and a sample (0.4 ml) was added to each of three Berkman-Schoefer cuvettes.⁹ Each cuvette contained prewarmed brain-heart infusion (15 ml; 1/3 strength).

Two cuvettes were used for tests and contained antibiotic, a different one in each. The antibiotics used are given in Table 1 and the concentration in the cuvettes in Table 2. In some cases, preliminary tests had to be carried out with standard organisms to ascertain the best concentration to use, but for most antibiotics suitable concentrations were already available.^{5,10} The third cuvette was used as a control for both tests and so contained no antibiotic.

Table 2 Concentrations of various antibiotics used in tests

Antibiotic	Concentration
Ampicillin	2.0 µg/ml
Gentamicin	0.5 µg/ml
Streptomycin	1.0 µg/ml
Tetracycline	0.25 µg/ml
Lincomycin	0.25 µg/ml
Erythromycin	0.06 µg/ml
Penicillin	0.1 U
Fucin	1.5 µg/ml
Methicillin	1.5 µg/ml
Carbenicillin	5 µg/ml
Mecillinam	1 µg/ml

Cuvettes were incubated and DLS curves recorded after periods of 30, 60, and 90 minutes. Before each curve was recorded the cuvette was allowed to rest in the chamber of the Differential I for about 1 minute. This procedure greatly reduced the noise level in the curves, probably because large particles settled to the bottom of the cuvette where they could not intercept the light beam. Not more than 20 tests could be performed during a 5-hour period. But this number could presumably be greatly increased by using the Differential III light-scattering photometer (Science Spectrum Inc, Santa Barbara, California, USA) which is an updated version of the Differential I, incorporating automatic sample changing.¹⁰

ANALYSIS OF DLS CURVES

The logarithm of the scattered intensity for the angular range 30°-130° was punched on to paper

tape, at 1° intervals, for analysis. For each sensitivity test the displacement index, D , and total score, S , were calculated.³ Also the difference between the logarithm of the intensity scattered at a fixed angle in a test and its control was calculated. Differences were calculated for angles of 30° , 60° , 90° , and 120° . All calculations were performed for the results obtained after each incubation period of 30, 60, and 90 minutes.

Noise-free curves were obtained by fitting a cubic spline to all experimental curves.⁶ Every calculation was repeated using these cubic spline representations in place of the curves obtained experimentally.

Fig. 1 Graph of variation in percentage disagreement between agar plate results and displacement index, D (▲), and the total score, S (●), after 30 minutes' incubation, against trial boundary level. The experimental DLS curves were used for (a) and their cubic spline representations for (b).

Results

Figure 1 shows that 30 minutes is an insufficient incubation period. It did not matter whether the experimental DLS curve or its cubic spline representation was chosen, or whether D and S was chosen as the index. The graphs of disagreement between agar plate and DLS tests against trial boundary levels showed no minima. Thus, there was no optimum boundary level for the DLS tests.

After an incubation time of 60 minutes the DLS results are more useful; once again the simple index, D , proved useful as the total score, S , that is incorporating morphological information, made little difference to the outcome of the tests. In the curves shown in Fig. 2, the percentage disagreement between

Fig. 2 Graph of variation in percentage disagreement between agar plate results and displacement index, D (●), and the total score, S (■), after 60 minutes' incubation, against trial boundary level. The experimental DLS curves were used for (a) and their cubic spline representations for (b).

agar plate and DLS test results is lower than in Figure 1. At the minima, the positions of which represent optimum DLS boundary levels, the dis-

agreement is just over 20%. In fact the disagreement is somewhat lower for D than for S. Once again the effect of replacing experimental curves by their cubic spline representations is negligible.

When the incubation time was increased to 90 minutes the agreement between DLS and agar plate tests was even better; but, as before, both D and S terms were equally useful, and the cubic spline representations were no more useful than the experimental DLS curves. Figure 3 shows that in all cases the level of disagreement fell to around 17% when the optimum boundary level was chosen.

Fig. 3 Graph of variation in percentage disagreement between agar plate results and displacement index, D (●), and the total score, S (■), after 90 minutes' incubation, against trial boundary level. The experimental DLS curves were used for (a) and their cubic spline representations for (b).

Why does S contain no more useful information than D? Figure 4 compares test and control DLS curves obtained for the action of lincomycin on a sensitive strain of *Staph. aureus*; it is apparent that the antibiotic causes the peak positions in the DLS curves to shift. The shaded area between the test and control curves in this figure is proportional to D. In Fig. 5 these curves have been scaled together so that the shaded area between them is now proportional to the morphological index, M. Comparison of the two figures shows that D is always much greater than M, so that, when the two are combined to compute S, the morphological index has little effect. D and S then convey essentially the same information.

Fig. 4 DLS curves from a sensitive strain of *Staph. aureus* in the presence of lincomycin (— — —) after incubation times of 30, 60, and 90 minutes. The hatched area between test and control (—) is equal to displacement index, D, multiplied by the angular range.

Figure 6 compares the results of the 60° single-angle tests with the agar plate results. Results obtained at 30°, 90°, and 120° were very similar, indicating that the chosen angle has little effect on the results of these tests. After an incubation time of only 30 minutes the disagreement was very high at around 40%. By 60 minutes it had dropped to around 27%. After 90 minutes the percentage disagreement had again fallen, this time to just below 20%. There was no noticeable effect resulting from the replacement of the experimental curves by their cubic spline representations.

Figure 7 shows why the problems anticipated by Stull⁵ are not important after an incubation time of 90 minutes. This figure compares test and control DLS curves obtained for the action of tetracycline on a sensitive strain of *Staph. aureus*. Because one effect of the antibiotic is to shift the peaks in the

Fig. 5 The same DLS curves as in Fig. 4 but with test and control curves scaled together so that the hatched area between them is equal to the morphological index, M , multiplied by the angular range.

DLS curve, a single-angle measurement at 60° would yield an 'insensitive' result because the test scatters no less light around this angle than the control after 30 minutes' incubation. However, in the total range 30°-130°, the test specimen clearly scatters less light. Thus, the single result is misleading. But after 90 minutes' incubation the effect of antibiotic is more marked and less light is scattered at a single angle of 60°, reflecting the behaviour throughout the angular range. Thus, the single angle measurement is no longer misleading.

Discussion

It is clear that, in our experience, the total score, S , introduced by Wyatt and his collaborators³ is of little more value than their simple displacement index, D , in sensitivity tests. An incubation period of 60 minutes would be adequate, but 90 minutes would lead to slightly more reliable results. The simple index is proportional to the difference in the

Fig. 6 Graph of variation in percentage disagreement between agar plate results and results based on light-scattering at a single angle (60°) against trial boundary level, after incubation times of 30, 60, and 90 minutes. The experimental DLS curves were used for (a) and their cubic spline representations for (b).

logarithm of the total intensity of light scattered, in the chosen angular range, by the test and control samples. Figure 4 makes the same point pictorially.

Consequently, there is no need to measure the angular dependence of the light scattered. The total scatter in the required range for the test and control would be sufficient to calculate D . This total scatter could be measured with a conceptually simpler instrument than the Differential I. With an incuba-

Fig. 7 Control (—) and test (---) DLS curves obtained for the action of tetracycline on a sensitive strain of *Staph. aureus* after incubation times of 30, 60, and 90 minutes.

tion time of 90 minutes an even simpler instrument which made a single-angle measurement would be adequate, although it might be preferable to make a few such measurements simultaneously and add the results. (Of course, if sufficient single-angle measurements are made the difference between the test and control sums will be proportional to D .) In practice, the simpler instruments which are used for antibiotic sensitivity testing are more complex than a simple nephelometer and are comparable in price to the Differential I.

How can we account for the 20% disagreement between DLS and agar plate tests? Some disagreement is inevitable when sharp, yet arbitrary, boundaries between 'sensitive' and 'resistant' are defined. In about one half of the cases of disagreement one method resulted in a score that was at the bottom end of the 'sensitive' scale and the other in a score at the top end of 'resistant'. We classify these as minor discrepancies.

There are three reasons for major discrepancies. A few cases were found where the agar plate method gave a 'sensitive' result, yet growth of the bacteria

was not affected by the antibiotic in a cuvette. A probable cause is an inactive antibiotic disc; in the agar plate tests this problem was overcome by placing the disc between test and standard organisms. A strictly analogous method is not possible in the DLS tests if antibiotic is added in the form of impregnated discs. But the problem could be overcome by not using discs and regularly testing batches of antibiotic against standard organisms. The second major cause of discrepancy was failure of the bacteria to grow. Here the DLS curves were characterised by a featureless, noisy appearance, and the intensity scattered by the control was, of course, much less than usual. This latter characteristic could be detected in a completely automated system and the test could be repeated. Finally, it must be remembered that the agar plate method can give a false result. In a trial, 114 errors were noted in 3386 tests.¹¹

The advantage of the DLS curve is that its form allows a reasonably intelligent guess as to the identity of the organism causing infection. This advantage is of obvious importance in the examination of cerebrospinal fluid from patients admitted with suspected meningitis. Identification of bacteria in this way has been proposed as another application of the DLS technique.¹² Furthermore, light-scattering photometers, such as the Differential I, are valuable research instruments for examining the structures of bacteria or indeed of any other particles of comparable size.

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