objective is to explore cell deformability as a physical characteristic in its own right, techniques using cells resuspended in artificial media (Gregersen et al., 1967; Weed et al., 1969; Forman et al., 1973; Schmid-Schönbein et al., 1973) or packed cell preparations (Jacobs, 1963; Dintenfass, 1965) can yield valuable information. However, as the physical properties of the red cells are almost certainly influenced by the surrounding plasma, we feel that the measurement of RBC deformability in whole blood is more clinically relevant. This appears to be borne out by preliminary studies of RBC deformability in patients with peripheral circulatory insufficiency (Reid et al., 1976).

In trying to reproduce conditions which prevail in the microcirculation the characteristics of the filter used are obviously critical. Several comparative studies of filtering devices have been published (Nicola et al., 1961; Gregersen et al., 1967; Forman et al., 1973; Rosenmund et al., 1975) and there seems to be general agreement that Nuclepore filters give results which are both sensitive and reproducible.

References


A rapid microbiological procedure using Bacillus stearothermophilus for the assay of antibacterial drugs

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The essential criteria for clinical assay procedures have been well summarized elsewhere (Phillips et al., 1974) as reasonable accuracy, simplicity, and speed both in technician time and in result. In 1971 Wahlig and Haemeister reported a method which seemed to combine these qualities with those of extreme sensitivity and the use of extremely small volumes of body fluids; this method was originally intended for gentamicin assays, and the present paper outlines the ways in which this idea has been developed. An unusual feature is that the assay indicator organism is Bacillus stearothermophilus, heavy inocula of which when incubated at about 60°C reach visible growth in three hours on a plate. One of us (HW) has developed the method in kit form1 with premeasured, dehydrated medium and gentamicin standards and a dried B. stearothermophilus spore suspension; the procedure was used under clinical conditions by Spring et al (1971), who found it to be highly satisfactory and to give excellent correlation with a standard microbiological method. The other (RJH) has adapted the principle as a daily routine test for amino-hexose and also for several β-lactam antibiotics as well as for erythromycin, chloramphenicol, lincomycin, chlorolincocin, fusidic acid, and the tetracyclines. The method has been used for clinical asays of each of these drugs.

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1 Refobacin-Test, Art. No. 11400, E. Merck, Darmstadt, Germany
Material and methods

(1) The kit form (Refobacin-Test, Merck, Darmstadt) is particularly suitable for occasional assays in the small hospital laboratory. It consists of three vials:

(a) contains sufficient dehydrated Merck antibiotic agar No. 5 for the preparation of 100 ml of agar medium by the addition of water and heating in a boiling waterbath for 20-30 minutes.

(b) contains a lyophilized suspension of *B. stearothermophilus* spores which are resuspended in 2 ml sterile water. When the medium prepared from vial (a) has cooled to 60°C, the 2 ml volume of spore suspension is added with thorough mixing, and it is recommended that 8 ml lots are poured into twelve 8 cm petri dishes; the plates should be dried at 37°C for 30 minutes.

(c) contains a predetermined weight of dried gentamicin base; 2 ml of serum, plasma or other appropriate body fluid is added to yield a standard of 8 μg/ml gentamicin base. A series of doubling dilutions are made with the body fluid, giving a final range of standards of 8, 4, 2, 1, and 0.5 μg/ml.

Thick filter-paper discs (Schleicher and Schüll, No. 2668), 6 mm in diameter, are supplied with the kit, and it is recommended that 0.02 ml of each standard and each body fluid under test should be pipetted onto the discs from either automatic or manual micropipettes. It is suggested that three discs of each standard and nine of each test fluid should be prepared, and that the resulting 24 discs should be distributed eight to each plate.

The plates are placed on a level grid in a waterbath at 60-62°C so that the water is just in contact with the lower face of the plates, or alternatively the plates can be floated on the water surface, thus ensuring complete contact. After three hours the diameters of zones of inhibition are measured with callipers or a zone-reader, the relevant means are calculated, and the standard graph is plotted; from this the concentration of gentamicin in the test fluid can be determined. Larger round or square plates can also be used for multiple assays.

Routine laboratory method

(a) *B. stearothermophilus* grows freely at 60°C on a wide variety of media; in the present study infusion broth and agar (Cruickshank, 1975) were used on most occasions, but diagnostic sensitivity test agar (Oxoid CM 261) or antibiotic-agar No. 5 (E. Merck, Darmstadt, Art. No. 5271) appear equally satisfactory.

(b) Much of the earlier experimental work was carried out with the strain of *B. stearothermophilus* supplied by Merck, but very similar results have since been obtained with the strain used by Oxoid in the preparation of their spore strips (BR 23) and the strains of the organism offered by the NCTC (10339, 10003, 10007). All appear to have comparable sensitivity to the antibacterial agents tested, and all grow at about the same speed provided young fresh suspensions are used.

A stock broth culture of the organism is maintained by daily inoculation into 5 ml broth, which is incubated overnight at 60°C. Either 14 cm diameter plastic petri dishes or 25 × 25 cm square Mast assay plates may be used for routine assays, although it is not easy to ensure that the larger plate is in full contact with the water surface during incubation. A base layer of infusion agar is poured into the plate, 25 ml for the 14 cm diameter plate and 150 ml for the square plate. When the base has set hard, an overlay of agar seeded with *B. stearothermophilus* is poured. One millilitre of overnight broth culture is added to 25 ml molten agar for the smaller plate, and 4 ml culture to 100 ml agar for the large assay plate, and the plates are dried for ½-1 hour at 37°C.

(c) Drug standards are prepared in serum, plasma or the appropriate body fluid whenever practicable. The range of standard strength is determined by the expected body fluid concentration and related clinical consideration; with gentamicin a range 0.5, 1, 2, 5, 10, 20 μg/ml has proved suitable while for benzylpenicillin 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 μg/ml is used. The indicator organism is so sensitive to several of the β-lactam antibiotics, notably benzylpenicillin, that it may be necessary to dilute the patient’s serum or body fluid 1/5 in normal human serum or body fluid.

The same 6 mm filter-paper discs can be used as drug reservoirs for standard and test fluids, and often 0.01 ml fluid gives zones of adequate size. Alternatively, No. 3 size fish spine beads (Lightbown and Sulitzeanu, 1957) have proved highly satisfactory as drug reservoirs provided they are free from chips and are from one batch of identical mould size; No. 3 beads hold approximately 0.025 ml serum.

The plates are incubated for 3 hours at 60°-62° as described in section (1), and the diameter (d1) of the zones of inhibition round both standard and test reservoirs are measured. The diameter d2 of the reservoir is also measured, and \((d_1 - d_2)^2\) value are plotted on the arithmetic axis of semi-log graph paper, with the drug concentration on the log axis. From the resulting standard curve, the drug concentration in the test fluid can be determined.
Results

Comparisons between results of gentamicin assays by the Rebofacin-Test and by a standard method (HW)

Sixty serum samples were tested by the kit method and by a standard agar diffusion method using B. subtilis ATCC 6633; the latter method was described recently by Wahlig et al (1975). Forty-one samples were also tested by both methods by Spring et al (1971).

The results from the earlier series are presented in the figure on which the calculated regression line has been drawn; the standard deviations of the results from both series are given in the table together with their correlation coefficients.

![Figure](image)

**Figure** Results of serum gentamicin assays by Rebofacin-Test plotted against those by a standard agar diffusion method.

High-temperature method as a routine procedure (RJH)

Reproducibility of assay

The same serum specimen was assayed for gentamicin by the high-temperature method on 10 occasions whenever routine assays were being performed during a three-week period; the serum sample was stored at -20°C between assays. A mean value of 5.44 µg/ml was obtained, the results falling between 5.1 and 5.7 µg/ml.

Similar results were obtained when a serum was assayed for benzylpenicillin on five successive days; the values lay between 6.4 and 6.91µg/ml with a mean of 6.66 µg/ml.

Comparison with other microbiological methods

(a) Results from gentamicin assays by the high-temperature method were compared with those obtained from two overnight agar diffusion methods using either Corynebacterium xerosis (Connaught Labs) or Staphylococcus aureus NCTC 6571, and a three-hour agar diffusion method using as indicator organism a fast-growing strain of Serratia marcescens which is very sensitive to gentamicin.

<table>
<thead>
<tr>
<th>Indicator organism</th>
<th>No. of assays</th>
<th>Range of corresponding results with B. steatorhemophilus method</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. xerosis</td>
<td>35</td>
<td>-9% to +12%</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>24</td>
<td>-11% to +8%</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>30</td>
<td>-12% to +14%</td>
</tr>
</tbody>
</table>

(b) Results from benzylpenicillin assays by the high-temperature method were compared with those from overnight agar diffusion assays using Staph. aureus NCTC 6571.

<table>
<thead>
<tr>
<th>Indicator organism</th>
<th>No. of assays</th>
<th>Range of corresponding results with B. steatorhemophilus method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td>8</td>
<td>-8% to +2%</td>
</tr>
</tbody>
</table>

(c) Erythromycin was assayed in sera by the high-temperature method and by overnight agar diffusion assays using Staph. aureus NCTC 6571.

<table>
<thead>
<tr>
<th>Indicator organism</th>
<th>No. of assays</th>
<th>Range of corresponding results with B. steatorhemophilus method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td>5</td>
<td>-7% to +3%</td>
</tr>
</tbody>
</table>
Discussion

Both variants of this procedure yield accurate drug assays after three hours' incubation, thereby fulfilling the prime purpose of the rapid assay, which is to permit the adjustment of the next four-, six- or eight-hourly dose should this be necessary. The results quoted show that the method is as accurate as those from other rapid plate assay methods with the advantage over these methods that relatively large zones of inhibition appear round even the lowest standard drug reservoirs; for gentamicin, the method is more accurate in the lower concentration area around 1-3 μg/ml than in the higher range of 6-10 μg/ml. Paradoxically, perhaps the greatest disadvantage of the method is the wide spectrum of sensitivity of the indicator organism, which often, therefore, precludes the possibility of differential assays when two or more antibacterial drugs are being administered simultaneously. Many β-lactam antibiotics can be inactivated with the appropriate β-lactamase, and p-amino benzoic acid can be incorporated in the medium to inactivate sulphonamides. B. stearothermophilus is very sensitive to lincomycin and chlorolincrocin as well as to gentamicin, tobramycin, and sisomicin, and members of these two groups are frequently given in combined therapy. In this situation the high-temperature method is unsuitable, and rapid plate assays of the aminohexose component require a different indicator organism; strains of Klebsiella or S. marcescens are suitable. Lincomycin and chlorolincrocin in the presence of aminohexoses are routinely assayed at Carshalton with a strain of haemolytic streptococcus Lancefield group A, which is very sensitive to the former but virtually resistant to aminohexoses.

Many workers in the United Kingdom customarily assay blood samples for gentamicin at the expected peak and also at trough, just before the injection. Peak levels usually occur between 20 and 80 minutes after intramuscular injection, according to the age and body weight of the subject, although clearly other factors may be involved; the rapid assay method permits adjustment of the subsequent dose even when the drug is being given at four-hourly intervals in very severe infections.

In practice, waterbath incubation at 60° for three hours has proved to be no problem, although prolonged incubation under these conditions may result in excessive condensation within the closed plate; it may be possible to incubate sealed plates in a dry incubator at 60°, and plates can also be incubated in the waterbath without a lid for up to 8 hours, after which they start to dry up. Plates to which filter paper drug reservoirs have been applied can be incubated dry at 60° for about four hours if they are placed bottom up in the incubator. The growth of laboratory contaminants at this temperature is extremely unlikely, and conventional aseptic precaution need no longer be employed. The method is particularly useful when urine and some other fluids are received in the laboratory already contaminated with Gram-negative bacteria, often of faecal origin. None of these troublesome contaminants, particularly Proteus spp, will grow at 60°, and even aqueous extracts of faeces have been successfully assayed.

References


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