Technical methods

Quality control in agar-dilution sensitivity-testing by direct assay of the antibiotic in the solid medium

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When antibiotic sensitivity of microorganisms is tested by agar dilution, single antibiotics are incorporated in solid media and 'spot' inocula of test strains are applied to the antibiotic media and to a plate without antibiotic. The usual method of quality control is to include control strains of known sensitivity.[1] However, control strains are usually sensitive to a number of antibiotics, and the absence of growth does not indicate which antibiotic is in a particular plate. In addition, strains chosen for controls are often either very sensitive or highly resistant to antibiotics so that they do not indicate moderate discrepancies in antibiotic concentrations.

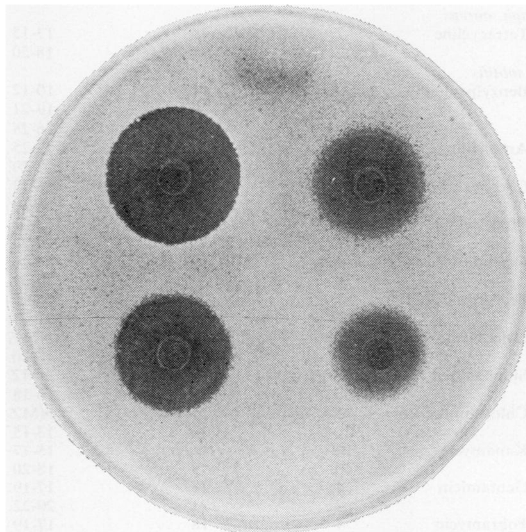
The direct assay of the antibiotic content of the solid media described here shows that each antibiotic is present at the correct concentration and provides some check on the identity of the antibiotic.

Material and methods

Antibiotic sensitivity was tested by Buckle's method of agar dilution.[2] The medium used for quality control tests was Wellcotest Sensitivity Test Agar (Wellcome), prepared every two weeks, dispensed in accurate 18 ml quantities into 9 cm petri dishes, allowed to set on a level bench, and stored at 4°C.

The indicator organisms used to check that the antibiotic sensitivity plates contained the correct antibiotics at the correct concentrations were an Alfred Hospital isolate of *Escherichia coli* known to be sensitive to trimethoprim, colistin, and nalidixic acid for plates containing these antibiotics, *Staphylococcus aureus* (ATCC 6538P) for tetracycline plates, and *Bacillus subtilis* (NCTC 8236) for all other antibiotic plates. *E. coli* and *Staph. aureus* were used as overnight tryptic digest broth cultures diluted 1 in 10 000 and 1 in 1000 respectively. *B. subtilis*, stored at 4°C as a spore suspension in saline (10^9 viable spores/ml), appeared to last indefinitely and was diluted 1 in 1000 for use. These dilutions allowed a dense but not quite confluent lawn to develop on incubation. Dried plates of quality control test medium were flooded with the above dilutions of the indicator strains, excess liquid was removed with a sterile Pasteur pipette, and the seeded medium was allowed to dry.

After the antibiotic plates had been inoculated by means of a multipoint inoculum replicator with the organisms whose sensitivity was being tested, a size 3 (7 mm) cork-borer was used to punch a plug in each antibiotic plate between the inoculum points. The agar plugs were removed with a blunt-tipped straight wire, and a maximum of four plugs per plate was placed on the surface of the seeded quality control media. The plates were incubated overnight at 37°C, and zones of inhibition around the antibiotic agar plugs were measured with calipers and examined for appearances of the zone edges (Figure).



Zones of inhibition showing different zone edges produced by antibiotic agar plugs. Indicator organism: B. subtilis. *Starting from top left hand corner and reading clockwise: Methicillin 10 μg/ml, lincomycin 10 μg/ml, chloramphenicol 20 μg/ml, kanamycin 20 μg/ml.*

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Results

ZONE EDGE

Inhibition zones on quality control plates with *E. coli* as indicator were clear and had clearly defined edges, the zones caused by trimethoprim and nalidixic acid being larger than those of colistin. With *Staph. aureus* as indicator, zones caused by tetracycline were a little less clearly defined than those on the *E. coli* plates. The zone edges with *B. subtilis* depended on the antibiotic in the agar plug (Figure). Benzylpenicillin, ampicillin, carbenicillin, methicillin, and cephaloridine caused clear zones with clearly defined edges; erythromycin and lincomycin caused double-edged zones; sulphadiazine and chloramphenicol zones showed a very hazy edge; kanamycin, gentamicin, and tobramycin caused clear zones but the edges were less clearly defined than those of the β-lactam antibiotics; and nitrofurantoin also caused a zone with a less clearly defined edge but smaller than those caused by the aminoglycosides.

Diameters of inhibition zones around antibiotic agar plugs

Indicator organism and antibiotic	*Concentration (μg/ml)*	*Mean zone diameter (mm)*	*Range (mm)*
E. coli			
Trimethoprim	1	16	15-17
	10	27	26-28
Nalidixic acid	12·5	12	11-13
	50	21	20-22
Colistin	4	NMZ	NMZ
	8	7	7-8
Staph. aureus			
Tetracycline	2	14	13-15
	5	19	18-20
B. subtilis			
Benzylpenicillin	0·1*	11	10-12
	1*	20	19-21
	10*	27	26-28
Ampicillin	5	24	23-25
	12·5	28	27-29
Carbenicillin	100	27	26-28
	200	29	28-30
Methicillin	4	19	18-20
	10	23	22-24
Cephaloridine	5	25	24-26
	20	31	30-32
Erythromycin	1	16	15-17
	5	22	21-23
Lincomycin	1	NMZ	NMZ
	10	19	18-20
Sulphadiazine	50	NMZ	NMZ
	100	17	16-18
Chloramphenicol	5	NMZ	NMZ
	20	14	13-15
Kanamycin	10	16	15-17
	20	19	18-20
Gentamicin	4	18	17-19
	8	21	20-22
Tobramycin	4	18	17-19
	8	21	20-22
Nitrofurantoin	30	NMZ	NMZ
	300	15	14-16

*units/ml
NMZ = no measurable zone

ZONE SIZE

The sizes of inhibition zones around antibiotic agar plugs were characteristic of antibiotics and their concentrations. The zone diameters shown in the Table are based on 80-100 measurements, but readings were consistent enough to have given the same results after 20-30 measurements. Zones with hazy edges were measured by ignoring the haze and measuring to the point at which there was no effect on the growth of the indicator organism. A difference of more than 2 mm from the mean zone diameter indicated an error in either the antibiotic or its concentration except for colistin which caused small zones of inhibition and where a difference of more than 1 mm in diameter indicated an error in concentration.

Initially, all concentrations of all antibiotics were tested for quality control every time sensitivity tests were performed. Later, when a high level of confidence was established between the personnel who prepared the antibiotic media and the benchworkers who used them, only one concentration of each antibiotic was tested when the first set of plates was used from the new batch of antibiotic media prepared each week. The lower concentrations were sampled for the penicillins and cephaloridine and the higher concentrations for all other antibiotics.

Discussion

Quality control of antibiotic media for agar dilution sensitivity tests is usually monitored by using control strains that are very sensitive to several antibiotics and very resistant to others. Such strains do not give precise information as to which antibiotic or what concentration of antibiotic is in a plate. Monitoring can be improved by using a selection of control strains of intermediate sensitivity.[3] However, an adequate number of 'intermediate' strains to cover all concentrations of all antibiotics is difficult to collect, time-consuming to maintain and, by occupying multipoint inoculator sites, reduces the number of sites available for test strains.

The direct assay quality control described in this paper uses a stable spore suspension of *B. subtilis*, requires only the maintenance of *Staph. aureus* and *E. coli* cultures, and, in addition, frees all inoculum sites for test strains.

Errors that may occur during the preparation of antibiotic media include a wrong calculation or wrong measurement during the preparation of antibiotic dilutions, loss of potency of an antibiotic, or omission of antibiotic. Zone size measurements in

this direct assay quality control detect all of these errors. In addition, if the wrong antibiotic is put in a prelabelled plate, the error may be detected if the observed zone size and/or zone edge differ from those that were expected.

The spore suspension is particularly convenient, and it had been hoped that it could be used for the assay of all antibiotics except colistin. However, all three strains of *B. subtilis* tested were found to be resistant to tetracycline as well as colistin and alternative assay organisms were therefore required for these two antibiotics. *E. coli* was selected for the assay of colistin but it was not suitable for the assay of tetracycline owing to the production of very small hazy zones. *Staph. aureus* was therefore selected for the assay of tetracycline. Although agar plugs containing trimethoprim and nalidixic acid caused inhibition zones with *B. subtilis* as indicator organism, *E. coli* was preferred for these antibiotics because the inhibition zones were larger and clearer.

Agar-dilution sensitivity-testing appears to be less subject to error than diffusion methods, and, with the direct assay quality control described here, it achieves a very high degree of reliability.

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Demarcation of antigen preparations on object slides in the immunofluorescent antibody test

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Applications of the immunofluorescent antibody technique are essential in virology, bacteriology, parasitologic serology, and immunohistopathology[1-6]. If background staining is not used the specimens in this technique are often colourless and invisible and therefore difficult to locate on object slides.[7] To overcome this difficulty we undertook a search for suitable methods for routine and research purposes. To test our method we applied it to the immunofluorescent antibody test using six different antigen preparations.

The antigen preparations were bordered on object slides with rings of paint, 3-7 mm in diameter (Fig. 1a). The paint was a two-component, water-soluble, chloric caoutchouc paint, Akva Epirex (Teknos-Maalit, Takkatie 3, 00370 Helsinki 37, Finland) and it was applied on the slides using a thin-walled metal cylinder of appropriate diameter (Fig. 1b). Object slides, equipped with rings, were used in the investigation of antibody-coated bacteria, toxoplasma and herpes antibodies, and antinuclear and other autoantibodies. Furthermore, the method was utilised in the search for tissue-bound immune reactants in frozen sections of human renal biopsies.

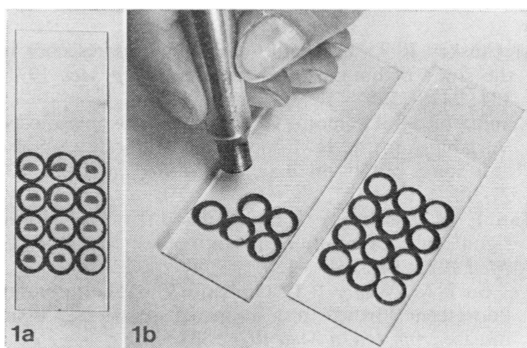

Fig. 1(a) *Object slide (2·5 × 7·6 cm) with rings. Bordered by the rings are cryostat sections of rat kidney, stained for photographic purposes with toluidine blue.* (b) *Application of the rings by labelling with a thin-walled metal cylinder.*

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