A Simple Plate Method for Multiple Tests of the Antibacterial Activity of many Bacteria against other Bacterial Strains

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SUMMARY: The organisms whose antibacterial powers are to be investigated are grown in parallel streaks on cellophan superposed on an agar plate. The cellophan is stripped off and the test organisms are streaked on the sterile agar surface thereby revealed, at right angles to the first streaks. After incubation, growth of the test organisms will be interrupted in those areas in which an inhibitor has diffused through the cellophan from the first organisms. There are some limitations in the application of the method.

During some recent work (Heatley & Florey, 1946) a strain of *Bact. coli* (*Escherichia coli*) was found to produce an antibiotic which inhibited some coliform organisms at a very high dilution, other coliform organisms being unaffected. Examination of a number of other strains of *Bact. coli* (M. A. Jennings, unpublished) showed that a few of them also formed antibiotics which were highly specific in the same way, inhibiting some strains of coliforms but not others, but differing from each other and from the first inhibitor examined in regard to the particular strains against which they were active.

The fact that the usual methods of detecting antibiotic action, employing only a few different strains, revealed a number of different highly specific inhibitors, suggested that there might be inhibitors formed by other strains of *Bact. coli* which had escaped detection because the test organisms used were not susceptible.

A simple test was required by which several strains of *Bact. coli* could be tested for antibacterial activity against several different test organisms. The method described below, which embodies more or less well-known principles (e.g. Gratia, 1944), was found to work satisfactorily, and may have wider applications, not only for the detection of antibiotic activity or the comparison of range of antibacterial action, but perhaps also for the selection of strains of bacteria which have special nutritional requirements, or of those which form an excess of certain growth factors, etc.

Principle of the method

The surface of a rather deep nutrient agar plate is covered with a circular sheet of cellophan and is then dried. The organisms to be tested are sown in parallel streaks on the surface of the cellophan (primary streaks), and the plate is incubated for 16–20 hr. (Pl. 1A). The cellophan, together with the organisms growing on it, is stripped off, and the test organisms (secondary streaks) are then sown across the surface of the agar at right angles to the position formerly

occupied by the primary streaks. After further incubation, the test strains will appear as in Pl. 1 B; the growth of the secondary streak is interrupted to a greater or less extent where it crosses the site of any primary streak which had produced an inhibitor against it. In fact, the plate itself presents the results of the test in tabular form.

Technical details

Depth of the agar. The agar should be rather deeper than usual, to prevent the slight inhibition of growth due to local exhaustion of the medium. About 100 ml. of nutrient agar/6 in. Petri dish is a convenient amount, giving a layer of medium 5–6 mm. deep.

Cellophan disks. These should be cut dry, slightly smaller than the surface of the agar, from cellophan about 0.03–0.04 mm. thick. A cellophan which gave satisfactory results with this technique was Cellophane grade P.T. 400 (British Cellophane Ltd., Bath Road, Bridgwater, Somerset). The waterproofed variety of cellophan cannot be used as it is not sufficiently permeable. The disks are washed in distilled water, and several of them may be autoclaved in a Petri dish with sufficient water to prevent them sticking together or becoming warped during sterilization.

Preparation of the plates. A sheet of sterile cellophan is picked up in flamed *but cool* forceps, the excess moisture is allowed to drain off, and the disk is spread out on the agar with forceps and, if necessary, a sterile bent glass rod. If the cellophan is not lying flat, e.g. because it has been gripped with hot forceps, it will gradually curl up during the drying and the plate will be spoiled. The plate should be well dried in any of the usual ways. Laying the lid and inverted bottom separately on parallel glass rods held in a frame in a 37° incubator for 1–2 hr. is satisfactory.

Making the streaks. The primary streaks should not be closer together than about 1.5 cm., so that six can be accommodated with ease on a 6 in. plate. The secondary streaks may, however, be closer together—0.8–0.9 cm. apart is a convenient distance—so that eleven may be made on the same 6 in. plate. The primary streaks should not be made nearer than 0.5–0.7 cm. to the edge of the cellophan, and if the latter shows a 'grain', i.e. irregular fine parallel lines presumably caused during extrusion, they are made parallel to the grain. The plates should be dried for a short time after streaking, otherwise there is a tendency for the bacterial growth to spread.

It is convenient for streaking to place the plate over a paper disk on which guide lines, the appropriate distance apart, have been boldly marked. By adopting such a standard procedure, the exact position of the primary streaks may be recalled at any time during or after the remaining operations.

The following method of making the streaks has been found much more convenient than the usual procedure with a loop. The sealed tip of a Pasteur pipette with a long thin capillary is cut off square, the open end flame-polished by holding in the base of a Bunsen flame, and the whole flame-sterilized. The capillary is then heated about 1 cm. from the end and allowed to bend through

nearly a right angle by its own weight. After cooling for a few seconds, the open tip is momentarily immersed in a broth culture of the organism which is to be streaked, and withdrawn before the meniscus has ceased to rise. Using the tip of the capillary like a pen, a metre or more of uniform streak—either continuous or in short lengths—can be traced without having to recharge the capillary (cf. loop technique). When all streaks with that particular organism have been made, the tip of the capillary is cut off just above the highest point to which the broth culture reached, and after flaming and bending, it is ready for the next culture. When the capillary has been used up, another is drawn out from the same pipette. This method (which surely must have been used many times before, though no reference to it could be found) gives a streak which is much more uniform throughout its length than can usually be obtained with a loop.

Limitations of the method

There are certain obvious limitations of the method:

(1) The technique cannot be used for the detection or study of inhibitors which will not diffuse through cellophan, e.g. the products of *B. brevis* (Dubos, 1939), or the protein diplococcin (Oxford, 1944). It may be possible to use instead of cellophan a membrane with larger pores, but still impermeable to bacteria, such as a gradocol membrane or cellophan treated in the manner described by Seymour (1940).

(2) In its present form it cannot be used unless primary and test organisms will both grow on the same medium. The medium used must also, of course, be one on which the antibiotic can be formed.

(3) The method is not suitable for organisms which spread on solid media. Partial success in dealing with such organisms was, however, attained by confining the streaks within ridges of beeswax-plus-resin or beeswax-plus-Venetian turpentine, the wax mixture being applied while hot with a bent Pasteur pipette, in the same way as the bacterial streaks.

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B. Same plate showing fully grown streaks of various test organisms, planted after removal of cellophan sheet.

A. Primary streaks of six different coliform organisms, on cellophan.