Studies on the 'carry-over' of antibiotics using the cellophane transfer technique

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SYNOPSIS The cellophane transfer technique has been used in the clinical laboratory as a guide to combined treatment and also to study the bactericidal actions of all common antibiotics, but during recent work it became clear that some antibiotics are absorbed on to the cellophane and enough may be carried through on to the fresh medium to inhibit growth of survivors even after transfer. The present investigation has been made to determine the maximum concentrations which can be used without risk of inhibition by carry-over. Certain concentrations are recommended and also the depth of agar, and with this there should be no risk of invalidating these bactericidal tests by bacteriostatic inhibition of survivors of carry-over. In clinical studies it is usually only necessary to test one of each group of antibiotics. That showing least carry-over should be used.

The cellophane transfer technique (Chabbert, 1957) consists of applying cellophane tambours (Fig. 1) inoculated with a suspension of the test organism to plates in which antibiotics have been pre-diffused from blotting paper strips. After incubation for six to 18 hours the tambour is transferred to antibiotic-free medium to permit growth of any organisms which have been inhibited but not killed. The method has the advantage that the entire bacterial population can be transferred and if two antibiotics are used and the strips set at right angles, it is possible to see the effect of the antibiotics alone and together over a wide range of concentrations.

FIG. 1. *Tambour after incubation following transfer to antibiotic-free medium.*

Received for publication 5 February 1965.

Chabbert and Patte (1960) used this method in the clinical laboratory as a guide to combined treatment and Garrod and Waterworth (1962) included it in their study of the bactericidal action of combinations of all the common antibiotics. Chabbert and Acca (1964) also applied the technique to rifamycin and the staphylomycin-pristinamycin group to relate antibacterial effect to biochemical mode of action.

METHODS

1 CELLOPHANE TRANSFER The procedure is as follows:
a Preparation of culture plates Blotting paper strips, 0.5×5 cm., are immersed in antibiotic solution, the excess is removed, and the strips are placed at right angles to each other on plates containing 30 ml. nutrient agar. These are incubated overnight at 37°C . and the strips removed.

b Preparation of tambours This work has been done with cellophane 300 MSAT or PT 300. These were chosen because they are commercially available as jam-pot covers and because they are the thinnest available (0.02 mm.): increase in thickness was found to increase carry-over. A cellophane square, 15×15 cm., is boiled in distilled water and tightly stretched over a pyrex glass ring, 8 cm. in diameter and 2.5 cm. high, and held in position with a strong rubber band. This tambour is placed in a glass dish 10 cm. in diameter, with the cellophane resting on a layer of moist filter paper and sterilized by autoclaving for 20 minutes at 120°C . The tambours should not be allowed to become dry.

c Preparation of cultures The tambour is placed on the plate into which the antibiotics have been pre-

diffused and its cavity flooded with about 2 ml. of an overnight broth culture of the test organism suitably diluted and the excess pipetted off.

d Transfer After incubation at 37°C. for six or 18 hours the tambour is transferred to a fresh plate containing 30 ml. agar and incubation continued for a further 18 hours to permit the growth of survivors.

Interpretation is according to Chabbert and Patte (1960) and Garrod and Waterworth (1962).

2 MODIFICATIONS OF THE CELLOPHANE TRANSFER TECHNIQUE

These modifications were made to observe carry-over.

a Paper strips Strips were prepared as above using concentrations of antibiotics ranging from 3,200 to 50 µg./ml. and applied to agar plates containing 15 or 30 ml. agar. After overnight incubation at 37°C. the strips were removed and an uninoculated tambour placed on the plate and incubated for six (P.M.W.) or 18 (Y.A.C.) hours. The tambours were then transferred to plates containing 15 or 30 ml. antibiotic-free medium and inoculated with a 1 in 1,000 dilution of an overnight broth culture of the Oxford H *Staphylococcus aureus* (209 P) which is sensitive to all the antibiotics studied: this dilution was chosen to give discrete colonies thus obtaining maximum sensitivity. Cultures were incubated at 37°C. overnight and any inhibition noted.

b Agar dilution (P.M.W.) Uninoculated tambours were placed on agar plates containing known amounts of antibiotics. After six hours' contact at 37°C. they were transferred to plates containing 15 or 30 ml. antibiotic-free agar and the tambours inoculated with staphylococci as above. Any inhibition of growth after 18 hours' incubation was noted.

c Determination of antibiotic concentration in agar after diffusion from paper strips (Y.A.C.) This was done by the method described by Klein (1953) and Hoette and Struyk (1957) which consists of cutting small cylinders of agar from the area into which the antibiotic has diffused and placing them on plates prepared as for assays. The zones of inhibition are compared with those from similar cylinders cut from plates containing known concentrations of antibiotics.

RESULTS

Using strips impregnated with different concentrations of 18 antibiotics (method 2a) we have determined the highest concentration of each antibiotic which does not inhibit growth of a sensitive staphylococcus by carry-over. The results of six hours' contact using plates containing 15 and 30 ml. agar and 18 hours' contact using 30 ml. plates are given in Table I; only full growth is included though there is often partial growth at higher concentrations.

The most striking observation is that the risk of carry-over is much greater when the plates contain

TABLE I
HIGHEST CONCENTRATION OF ANTIBIOTICS GIVING NO CARRY-OVER¹

	<i>Method 2a: Blotting Paper Strips</i>			<i>Method 2b: Dilution in Agar</i>	
<i>Contact time (hr.)</i>	6	6	18	6	6
<i>Transfer on (ml.)</i>	15	30	30	15	30
<i>Antibiotic</i>					
Benzylpenicillin	50	400	200	2	8
Methicillin	1,600	3,200	1,600	64	256
Oxacillin	400	1,600	400	8	64
Cloxacillin	400	3,200	200	8	64
Ampicillin	200	800	3,200	4	32
Streptomycin	1,600	3,200	3,200	64	256
Neomycin	50	100	200	2	8
Kanamycin	100	1,600	1,600	16	64
Chloramphenicol	3,200	3,200	3,200	128	256
Tetracycline	100	200	200	4	16
Oxytetracycline	200	800	400	8	64
Demethylchlortetra	50	50	100	2	4
Erythromycin	100	400	400	8	32
Spiramycin	3,200	3,200	1,600	128	256
Novobiocin	100	200	100	4	16
Fucidin	50	100	50	1	16
Rifamycin	50	100	50	2	16
Vancomycin	1,600	3,200	1,600	64	256

¹Results are expressed in µg./ml.

only 15 ml. agar, and it is clear that the concentration must be selected with the depth of the agar plates used in mind. Increasing the period of contact from six to 18 hours does not greatly increase carry-over.

The results of placing tambours on plates containing known concentrations of antibiotics (method 2b) show good correlation with the strip method (2a) and again show the importance of having a sufficient depth of agar to permit dilution of any antibiotic carried over.

Some tests were also done with polymyxin B and colistin. It was clear that both are heavily absorbed onto cellophane but results were very erratic and this technique cannot be recommended for use with polypeptide antibiotics.

The amount of antibiotic in the strips should be such as will give a gradient in agar comparable to that attainable *in vivo*. Such concentrations of the 18 antibiotics are given in Table II and were selected by direct determination of the concentrations attained in the surrounding medium after 18 hours' diffusion.

DISCUSSION

All the concentrations recommended in Table II are below the highest found to be free from carry-over and shown in column 3 of Table I. If strips are impregnated with 600 µg./ml. benzylpenicillin, sufficient is carried over to inhibit the growth of a sensitive staphylococcus, but if the test organism is a group D streptococcus requiring about 2 µg./ml. to inhibit it, survivors grow freely. This concentra-

TABLE II
ANTIBIOTIC LEVELS IN AGAR AFTER DIFFUSION
FROM PAPER STRIPS

Antibiotic	Concentration of Solution in Strips ($\mu\text{g./ml.}$)	Concentration in Agar 1 mm. from Strip ($\mu\text{g./ml.}$)
Benzylpenicillin	600	20
	50	1
Methicillin	1,000	20
Oxacillin	200	10
Streptomycin	500	20
Kanamycin	500	20
Chloramphenicol	1,000	30
Tetracycline	200	8
Erythromycin	200	6
Spiramycin	2,400	40
Novobiocin	100	2
Rifamycin	50	1
Vancomycin	400	20

tion, producing a gradient from 20 $\mu\text{g./ml.}$, is useful for testing penicillin-streptomycin combinations for the treatment of bacterial endocarditis.

When plates containing 30 ml. agar are used, only novobiocin, fucidin, and rifamycin are significantly carried over from strips impregnated with solutions of less than 200 $\mu\text{g./ml.}$, and for these a gradient of from 2 or 1 $\mu\text{g./ml.}$ may have to be used. In practice these tests are usually only done with organisms which are more resistant than a sensitive staphylococcus, when it may be possible to increase the con-

centration. It is of interest that at least two of the antibiotics which are heavily absorbed onto cellophane are also known to be heavily bound to protein.

Marked differences in carry-over may be found between antibiotics which are closely related, e.g. penicillin and ampicillin, neomycin and kanamycin and demethylchlortetracycline and oxytetracycline. In clinical studies it is usually only necessary to test one of each group: that showing least carry-over should be used.

If the concentrations recommended here are used with plates of at least 30 ml. agar in 9 cm. Petri dishes, there should be no risk of invalidating these bactericidal tests by bacteriostatic inhibition of survivors by carry-over. On the other hand it is clearly important that this phenomenon should be fully investigated before any new substance is used with this technique, even if it is closely related to an already known one.

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