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

Y. A. CHABBERT AND PAMELA M. WATERWORTH

From the Institut Pasteur, Paris, and the Postgraduate Medical School, London

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. This study 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.

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 Acet (1964) also applied the technique to rifamycin and the staphylococcal-intrinsic 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 x 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 FT 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 x 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 con-
taining 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 TECH-
nique 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 agar plates
containing 15 or 30 ml. antibiotic-free medium and
incubated 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 staphylo-
cocci 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 concentra-
tions of antibiotics.

RESULTS

Using strips impregnated with different concentra-
tions of 18 antibiotics (method 2a) we have deter-
mined the highest concentration of each antibiotic
which does not inhibit growth of a sensitive staphy-
lococcus 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
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 con-
taining 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 impreg-
nated 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 I |
| HIGHEST CONCENTRATION OF ANTIBIOTICS GIVING NO CARRY-OVER¹ |
| | Method 2a: Blotting Paper Strips |
| | Method 2b: Dilution in Agar |
| Contact time (hr.) | 6 | 18 | 6\* | 18\* |
| Transfer on (ml.) | 15 | 30 | 15 | 30 |
| Antibiotic | | | | |
| Benzylpenicillin | 50 | 400 | 200 | 8 | 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 |
| Démethylchlorotetra | 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 |
| Rifampicin | 50 | 100 | 50 | 2 | 16 |
| Vancomycin | 1,600 | 3,200 | 1,600 | 64 | 256 |

¹Results are expressed in µg./ml.
Y. A. Chabbert and Pamela M. Waterworth

TABLE II

ANTIBIOTIC LEVELS IN AGAR AFTER DIFFUSION FROM PAPER STRIPS

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration of Solution in Strips (µg./ml.)</th>
<th>Concentration in Agar 1 mm. from Strip (µg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>600</td>
<td>20</td>
</tr>
<tr>
<td>Methicillin</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>1,000</td>
<td>20</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>500</td>
<td>20</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>500</td>
<td>20</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1,000</td>
<td>30</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>200</td>
<td>8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>200</td>
<td>6</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>2,400</td>
<td>40</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Rifamycin</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>400</td>
<td>20</td>
</tr>
</tbody>
</table>

tion, producing a gradient from 20 µg./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 µg./ml., and for these a gradient of from 2 or 1 µg./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 concentration. 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.

REFERENCES