Detection of tetracycline resistance in *Streptococcus pneumoniae*

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SYNOPSIS  Bacteriological details are given of a patient with chronic purulent bronchitis, who was being followed up during a survey of relapse in chronic bronchitis. A strain of *Streptococcus pneumoniae*, serotype 10, was isolated from the sputum over a period of six months, followed by a type 47A strain and later a type 28 strain. The patient was receiving prophylactic treatment with tetracycline throughout. The type 10 strain was sensitive to tetracycline in vitro by both the disc diffusion and doubling dilution sensitivity tests and mice infected with this strain were protected by tetracycline. In contrast, both the type 47A and type 28 strains were sensitive by the disc diffusion technique, but showed a low degree of tetracycline resistance by the doubling dilution method; mice infected with both these strains were not protected by tetracycline.

During a survey of relapse in chronic bronchitis, strains of *Streptococcus pneumoniae* of varying serotypes were isolated from a patient who was receiving prophylactic tetracycline therapy. All the strains appeared sensitive to tetracycline using the routine disc diffusion sensitivity test.

This report concerns results of doubling dilution sensitivity tests and of mouse experiments on three of the serotypes.

Materials and Methods

The patient was a male, aged 51 years, with chronic purulent bronchitis. He was receiving prophylactic treatment with tetracycline, 250 mg four times a day, as an outpatient. He was being followed up as part of an investigation into relapse in chronic bronchitis. He was seen at the chest clinic every two months, when sputum was collected for culture. Sputum was also delivered to the laboratory every fortnight and at times of exacerbations.

Sputum Culture

The sputum was homogenized in sterile deionized water and the homogenate inoculated onto blood agar and heated blood agar plates. These were incubated at 37°C in an atmosphere of 10% CO₂ for 18 hours.

Strains of *Strept. pneumoniae* were identified by optochin sensitivity. All strains were typed by the capsule swelling technique using typing sera obtained from the Statens Seruminstitut in Copenhagen.

Sensitivity Tests

Two methods of testing for tetracycline sensitivity were used.

The disc diffusion method

Blood agar plates, using sensitivity test agar (Oxoid), were flood-seeded with an 18-hr serum broth culture of the test strains of *Strept. pneumoniae* or a 1 in 10 dilution of an 18-hr nutrient broth culture of the Oxford H strain of *Staphylococcus pyogenes* (as a standard sensitive organism). After drying, discs containing 10 μg tetracycline (Oxoid) were placed on the plates. After incubation at 37°C in 10% CO₂ for 18 hr, the width of the zones of inhibition of the test and the standard organisms (from the edge of the disc to the edge of growth) were measured with callipers. The results were expressed as the difference between the width of the zone of inhibition of the *Strept. pneumoniae* and that of the standard *Staph. pyogenes*. A strain was considered resistant if the zone of the test organism was at least 3 mm less than that of the standard strain.

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The doubling dilution technique

Tetracycline was incorporated in blood agar, using sensitivity test agar (Oxoid), in 9 cm petri dishes at final concentrations of 10.0 by two-fold dilutions to 0.015 µg per ml of medium. After drying, standard loopfuls (4 mm diameter) of the cultures used for the disc diffusion technique were streaked radially round the plate so that the standard strain of Staph. pyogenes and the various strains of Strept. pneumoniae were tested on the same plates. The plates were incubated as for the disc diffusion method and were read for 'growth' or 'no growth'. The results were expressed as resistance ratios, that is to say, the minimum inhibitory concentration (MIC) for the Strept. pneumoniae over the MIC for the standard Staph. pyogenes.

Mouse experiments

Three strains of different serotypes of Strept. pneumoniae were used to test for sensitivity to tetracycline in mice; they were a type 10, a type 47A, and a type 28. Groups of 16 mice were inoculated intraperitoneally with 10⁹ viable organisms per mouse as shown by viable counts. Half an hour after inoculation 1 mg of tetracycline was given subcutaneously to eight mice in each group; the remaining eight animals acted as untreated controls. The animals were checked daily and the heart blood and peritoneal fluid from any dead animals were cultured for Strept. pneumoniae. All cultures obtained were tested to confirm that the organisms recovered were of the same serotype as that inoculated into the animal. All surviving animals were sacrificed at eight days.

Results

Tetracycline sensitivity tests

The results of the two tests on the three serotypes of Strept. pneumoniae are shown in the table. All six type 10 strains were sensitive by both methods. The five type 47A strains and the six type 28 strains were sensitive by the disc diffusion method, but they showed a low degree of resistance when tested by the doubling dilution technique (resistance ratios of 8 and 4 respectively). Four of the five type 47A strains, isolated between 16 March 1971 and 13 April 1971, gave resistance ratios of 8 when re-tested on 22 April 1971 and on 16 June 1971; the first two strains were retested on 27 October 1971 with the same result. The type 28 strains were not retested.

Mouse experiments

The results are shown in the figure. Mice infected with the type 10 strain, which was sensitive by both sensitivity test methods, were protected by treatment with tetracycline; the mean survival time of the treated mice was greater than 6.75 days as compared with 0.13 days in the control, untreated mice. In contrast, mice infected with either the type 47A or type 28 strain, both of which showed a low degree of tetracycline resistance by the doubling dilution technique, had mean survival times of 0.75 and 0.85 days in the treated groups respectively and less than 0.13 days in the untreated groups in both cases.

Cultures of Strept. pneumoniae were recovered

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Period of Isolation</th>
<th>No. of Isolates</th>
<th>Tetracycline Sensitivity¹</th>
<th>Doubling Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disc-Width of Zone of Inhibition (mm)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td>10</td>
<td>24 August 1970–2 February 1971</td>
<td>6</td>
<td>6-8/6-8</td>
<td>0.4/0.4</td>
</tr>
<tr>
<td>47A</td>
<td>16 March 1971–26 April 1971</td>
<td>5</td>
<td>7-8/7-8</td>
<td>3.0/0.4</td>
</tr>
<tr>
<td>28</td>
<td>25 May 1971–13 September 1971</td>
<td>6</td>
<td>8-9/7-8</td>
<td>3.0/0.8</td>
</tr>
</tbody>
</table>

Table Results of sensitivity tests on cultures of Streptococcus pneumoniae

¹Reading for Strept. pneumoniae that for standard Staph. pyogenes.
²See text.
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from all the mice that died and they proved to be of the same serotypes as the infecting strains.

Discussion

The disc diffusion method of testing for antibiotic sensitivity of bacteria (Gould and Bowie, 1952) is widely used in routine bacteriological laboratories, since it is relatively quick and large numbers of cultures can be tested on a wide range of antibiotics. However, occasional cultures have appeared sensitive by this method but resistant by a doubling dilution technique (unpublished evidence), when the clinical findings suggested resistance to the drug.

The strains of Strept. pneumoniae reported in the present paper were isolated during a wider survey of patients with chronic bronchitis. In this survey all cultures of Strept. pneumoniae were serotyped, and were tested for sensitivity to tetracycline by both the disc diffusion and the doubling dilution techniques.

Two of the three serotypes reported were resistant by the doubling dilution method, although not by the disc diffusion method. Repeat testing up to seven months after isolation showed the resistance to be stable. The failure of tetracycline to protect mice infected with these strains and the fact that the patient was receiving prophylactic tetracycline over the period of isolation suggest that the resistance shown by the doubling dilution method was clinically significant.

The intensive follow up in the investigation mentioned concerned only six patients, including the one referred to in this paper. In none of the other five was a strain of Strept. pneumoniae of a low degree of tetracycline resistance isolated when the patient was not receiving tetracycline therapy. The clinical significance of the tests in vitro could not therefore be verified by the response to tetracycline treatment.

The third strain, serotype 10, was sensitive by both sensitivity tests. The mice infected with this strain were protected by tetracycline, although they were infected with $10^9$ viable organisms and only a single dose of 1 mg tetracycline per mouse was given. This suggests that the strain was truly sensitive to the drug. However, the patient was receiving tetracycline throughout the period of excretion of this type. It is possible that the drug was not being taken in the prescribed dose, as the patient was at home and the administration of the drug could not be supervised. One specimen of sputum did not inhibit Staph. pyogenes when tested by the cup diffusion assay technique. Alternatively, this strain may have been present as a sensitive 'persister', such as the organisms reported by MacDermott (1958), for which no adequate explanation exists.

Both of the resistant strains were of a 'low' degree of resistance. Previous work with tests for drug sensitivity in tubercle bacilli (Stewart, 1955; Stewart, Hall, Riddell, and Somner, 1962) showed that, when a sensitivity test proved unreliable, strains of 'low' degrees of resistance were the ones most likely not to be detected by the unsatisfactory test. Such strains of 'low' degrees of resistance were shown to be of clinical significance (Stewart and Crofton, 1964).

The results of the present work and of other unpublished results suggest that if a strain of bacteria appears sensitive by the disc diffusion technique, but clinical results suggest that resistance may be present, a doubling dilution test may detect such resistance.

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