

Further studies on the reliability of the bacitracin inhibition test for the presumptive identification of Lancefield group A streptococci

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SUMMARY The reliability of the bacitracin inhibition test to differentiate between 125 Lancefield group A and 122 non-group A beta-haemolytic streptococci was studied. Bacitracin discs containing 0.02, 0.04, or 0.1 international units and the conditions recommended by both the Association of Clinical Pathologists and the Federal Drug Administration for routine sensitivity testing were used. The results suggest that the presence or absence of a zone of inhibition around a 0.04 unit disc can be used routinely for the presumptive identification of group A streptococci and that a specific zone size can be used for reading the test only with a 0.1 unit disc.

Infections caused by beta-haemolytic streptococci of Lancefield group A can lead to rheumatic fever or glomerulonephritis. It is therefore important to distinguish this group from other beta-haemolytic streptococci. In 1953 Maxted reported a method for differentiating streptococci belonging to group A from other beta-haemolytic streptococci by the sensitivity of group A strains to bacitracin. Filter paper squares were soaked in a solution of bacitracin (5 international units per ml) and rapidly dried. The strain of streptococcus to be tested was streaked on the surface of a blood agar plate and a bacitracin impregnated filter paper square was placed on the inoculated area. After 24 h incubation at 37°C the plate was examined for zones of inhibition. When over 3000 strains were tested the method was found to identify 99.9% of group A strains. Levinson and Frank (1955), using a different medium and filter paper discs 6.4 mm in diameter, reported that for optimum performance the discs needed to be soaked in a bacitracin solution of only 1 unit per ml. They concluded that the bacitracin test was suitable for differentiating streptococci from respiratory sites whenever it was not feasible to use serological methods and also for conducting large-scale epidemiological investigations on respiratory disease.

However, since that time there have been varying opinions on the usefulness of this test. It has been performed in a variety of different ways with different inocula, media, disc contents, and interpretative

criteria and direct comparison of the results of different workers is impossible. In investigating the reliability of this test two main questions arise. First, are all the commercially available bacitracin discs suitable for routine testing? Second, what criteria should be applied in the interpretation of the test?

Bacitracin discs supplied by different manufacturers do not necessarily contain the same concentrations of bacitracin, and discs ranging from 0.02 to 0.2 international units per disc are available. Results obtained by Chitwood *et al.* (1969) indicate that differences in the bacitracin contents of discs can influence the reliability of the bacitracin inhibition test. Using the presence or absence of a zone of inhibition as the criterion for reading the test, they found unacceptable differences in the performance of discs obtained from four manufacturers.

Although Maxted (1953) originally used the presence or absence of a zone of inhibition to demonstrate bacitracin sensitivity, Washington *et al.* (1974), using a disc content of between 0.02 and 0.04 unit, successfully employed a specific zone size of 11 mm to differentiate group A from non-group A strains. On the other hand, Ederer *et al.* (1972), using discs containing 0.04 unit of bacitracin and the manufacturer's recommendations of a specific zone of inhibition of 10 mm, found that 6% of group A strains were not identified and 13% of non-group A streptococci were wrongly identified as group A, and Arvilommi (1976) falsely identified 43% of non-group A strains as group A using 0.2 unit discs and a

cut-off zone size of 10 mm recommended by the manufacturer.

This paper describes a comparison of the performance of bacitracin discs containing 0.02, 0.04, and 0.1 IU using the conditions recommended by both the Association of Clinical Pathologists (ACP) and the Federal Drug Administration (FDA) for routine antibiotic sensitivity testing. By using these standardised test conditions it is hoped to derive reliable interpretative criteria for the bacitracin inhibition test.

Methods

STREPTOCOCCI

A total of 247 strains of streptococci, which showed beta-haemolysis on Columbia agar (Oxoid) with 5% horse blood, were studied. Streptococcal antigen extracts were prepared by the autoclave method (Rantz and Randall, 1955) and were tested against streptococcal antisera (Wellcome Laboratories, Beckenham, Kent) by counter-current immunoelectrophoresis (Edwards and Larson, 1973).

BACITRACIN

Bacitracin powder, zinc salt, with a stated potency of 64 international units (IU) per mg and bacitracin discs containing 0.1, 0.04 or 0.02 IU were obtained as a special order from Scientific Hospital Services Ltd, Liverpool.

Bacitracin discs were also purchased from the following sources: Bacitracin Discs (Oxoid), Bacitracin Discs (Mast Laboratories), TaxoA (Baltimore Biological Laboratories, BBL), and Differential Discs (Difco Laboratories). All discs were stored at 4°C and the bacitracin powder was kept in a desiccator at 4°C.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) TO BACITRACIN

The MICs were determined by the agar dilution method (Ericsson and Sherris, 1971). A stock solution of bacitracin (100 IU per ml) was prepared by dissolving 100 mg of bacitracin powder in 64 ml of distilled water (a few drops of normal hydrochloric acid were added to effect a solution). Appropriate dilutions were prepared at 10 times the final concentration, and 2 ml of each dilution was added to 18 ml of molten Mueller-Hinton agar (BBL) containing 5% defibrinated horse blood at 56°C. The final concentrations ranged from 0.008 to 1.0 IU per ml (see Fig. 1). Streptococci were grown in Brain Heart Infusion Broth (Oxoid) enriched with 10% horse serum No. 5 (Wellcome) for 24 h at 37°C. Each culture was agitated on a Vortex mixer for 30s and diluted in saline to give a nephelometer

reading (Univalvo Type 200, Eel Ltd, Essex) similar to that of a barium sulphate (BaSO₄) standard (0.05 ml of 1% (w/v) BaSO₄ added to 99.5 ml of 1% H₂SO₄). Each plate was inoculated with about 0.001 ml of the streptococcal suspensions containing 10⁴-10⁵ colony-forming units (cfu) using an inoculating apparatus (Denley Ltd, Bolney, Sussex), and the plates were incubated at 37°C for 18 h.

SENSITIVITY OF STREPTOCOCCI TO BACITRACIN

(1) *Federal Drug Administration recommendations for routine sensitivity testing (NCCLS Subcommittee on Antimicrobial Susceptibility Testing, 1974)*

Streptococcal suspensions containing 10⁷-10⁸ cfu per ml were prepared as previously described and were inoculated onto Mueller-Hinton agar containing 5% horse blood using 'Q'-tips (Cheeseborough-Ponds Ltd, London). After a few minutes bacitracin discs (Scientific Hospital Services Ltd) were placed on the agar and allowed to prediffuse at room temperature for 15 min before the plates were incubated at 37°C for 18 h. Inhibition zones were measured with vernier calipers.

(2) *ACP recommendations for routine sensitivity testing (Stokes and Waterworth, 1972)*

Streptococci were grown in Brain Heart Infusion Broth with 10% horse serum at 37°C for 24 h, and a 5 mm loopful of each suspension was added to 3 ml of quarter-strength Ringer's saline to give a concentration of about 10⁵ cfu per ml. Diagnostic Sensitivity Test agar (Oxoid) containing 5% saponin lysed horse blood was inoculated by flooding the plate with a streptococcal suspension. Excess fluid was removed and each plate was dried at 37°C for 15 min. The bacitracin discs were applied as previously described and the plates were incubated at 37°C for 18 h.

EFFECT OF CARBON DIOXIDE ON THE SENSITIVITY OF STREPTOCOCCI TO BACITRACIN

The effect of carbon dioxide on the MICs and on the disc sensitivity tests of 24 strains of streptococci was studied by incubating the plates in a candle jar for 18 h at 37°C.

PERFORMANCE TESTING OF BACITRACIN DISCS FROM DIFFERENT SUPPLIERS

Mueller-Hinton agar containing 5% horse blood was poured on levelled glass assay plates (30 × 30 cm) to give an agar depth of 4 mm, and each plate was dried at 37°C for 15 min. A streptococcal strain with an MIC of 0.01 IU bacitracin per ml was selected and grown in Brain Heart Infusion

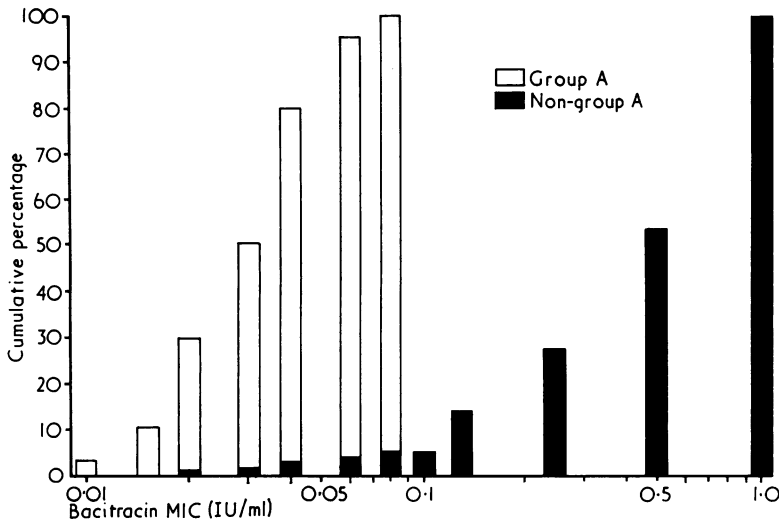


Fig. 1 Distribution of bacitracin MIC values in 125 strains of group A streptococci and in 122 strains of non-group A streptococci. Non-group A indicates strains belonging to groups B, C, D, and G.

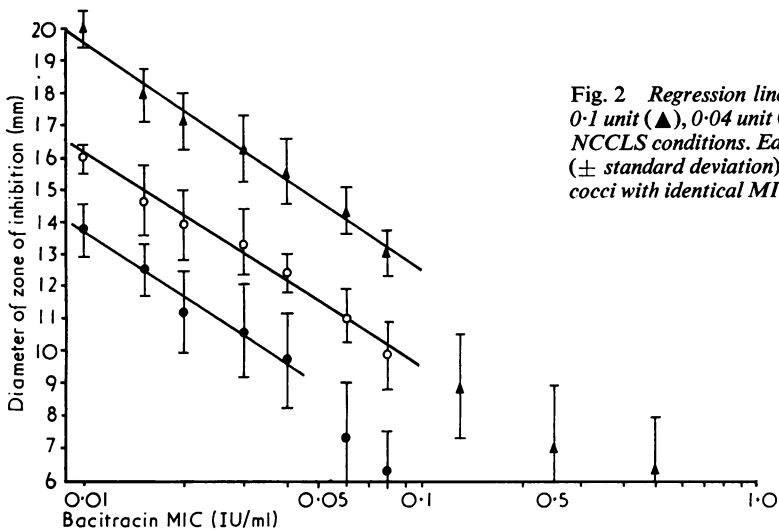


Fig. 2 Regression lines for bacitracin discs containing 0.1 unit (\blacktriangle), 0.04 unit (\circ), and 0.02 unit (\bullet) using NCCLS conditions. Each point represents the mean value (\pm standard deviation) of the zone diameters for streptococci with identical MIC values.

Broth containing 10% horse serum for 24 h at 37°C. The turbidity was adjusted to about 10^8 cfu per ml and 20 ml of this suspension was added to 180 ml of Mueller-Hinton broth (BBL). Each assay plate was inoculated by flooding the agar with 25 ml of this diluted suspension. Excess fluid was removed and the plate was dried at 37°C for 15 min. Fifty bacitracin discs of each concentration from Scientific Hospital Services and 50 discs from each of the other manufacturers were mixed in a sterile jar, and 100

discs were applied to each assay plate. The plates were incubated at 37°C for 18 h and inhibition zones were measured with vernier calipers.

Results

The distribution of MICs for bacitracin of the streptococci are shown in Fig. 1. All 125 Lancefield group A strains were inhibited by a concentration of 0.08 IU per ml but eight of the 122 non-group A

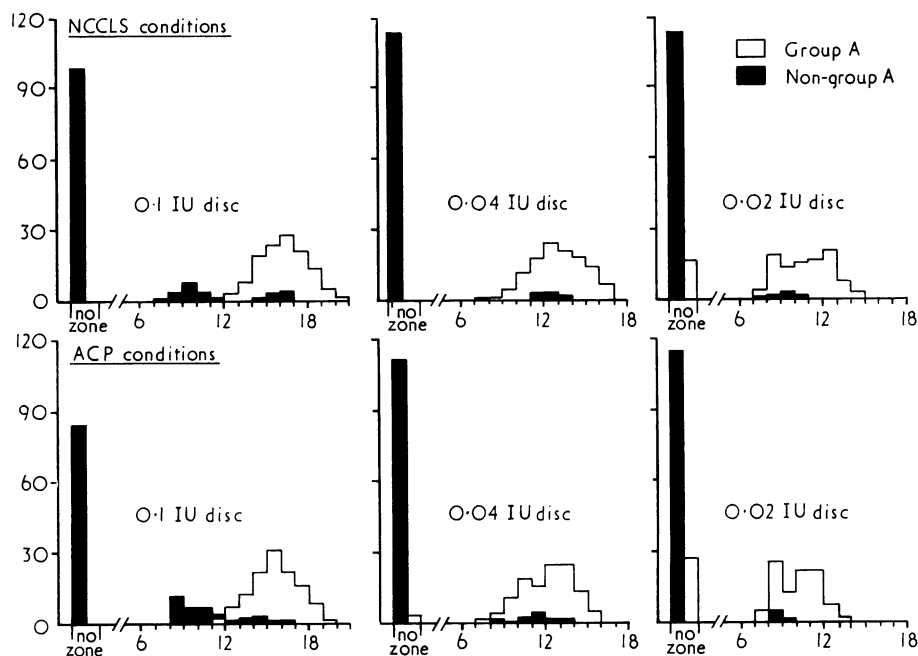


Fig. 3. Diameters of inhibition zone produced by 248 strains of streptococci against bacitracin discs of different concentrations using ACP and NCCLS conditions.

strains, one group B, two group G, and five group C strains were also inhibited by this concentration of bacitracin. Incubation in carbon dioxide did not alter the bacitracin MICs of the streptococci but it enabled easier reading of the end-points because of the heavier growth.

The regression lines were derived by plotting the mean diameter of the inhibition zones for each of the three bacitracin disc contents (0.1, 0.04, and 0.02 IU per ml) against the corresponding MIC (Fig. 2). In general there was a straight-line relationship between the MICs and zone diameters although for the 0.1 unit disc this relationship did not hold for MICs greater than 0.08 IU per ml.

The distribution of the inhibition zones for each content of bacitracin disc tested was similar using both the ACP and NCCLS test conditions (Fig. 3). A small proportion of non-group A strains had zones of inhibition which were identical with those of group A strains, and several group A strains were not inhibited by the 0.02 unit disc. Incubation in carbon dioxide did not alter the sizes of the inhibition zones of the 24 strains tested.

The results of assaying 50 bacitracin discs from each manufacturer and the standard discs from Scientific Hospital Services against a very sensitive group A streptococcus (MIC 0.01 IU per ml) are shown in Table 1. The bacitracin discs from com-

Table 1. Comparison of bacitracin discs from different manufacturers. Assay organism is a sensitive streptococcus, (MIC 0.01 IU per ml).

Bacitracin discs			Zone diameter (mm)		
Source	Concentration (IU)	Size (mm)	Mean	Range	± SD
SHS	0.02	6	16.5	15.8-17.0	0.6
SHS	0.04	6	18.4	17.9-18.9	0.7
SHS	0.1	6	20.4	19.1-21.2	0.6
BBL	0.04	6	16.9	16.2-17.8	0.5
Oxoid	0.05	6	17.2	16.0-18.0	0.6
Mast	0.1	6	22.0	21.0-23.0	0.7
Difco	NG	10	20.1	19.6-20.7	0.5

NG: not given; SD: standard deviation

Table 2 Zone or no zone interpretation of the bacitracin inhibition test

Disc concentration	NCCLS conditions				ACP conditions			
	Group A strains		Non-group A strains ¹		Group A strains		Non-group A strains ¹	
	Zone	No zone	Zone	No zone	Zone	No zone	Zone	No zone
0.1	125	0	24	98	125	0	37	85
0.04	125	0	9	113	122	3	8	114
0.02	107	18	8	114	98	27	6	116

¹Non-group A indicates strains belonging to groups B, C, D, and G.

Table 3 Reading of the bacitracin inhibition test using a minimum zone size of 12 mm inhibition.

Bacitracin disc concentration (IU)	NCCLS conditions				ACP conditions			
	Group A strains		Non-group A strains ¹		Group A strains		Non-group A strains ¹	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
0.1	125	0	8	114	123	2	8	114

¹Non-group A indicates strains belonging to groups B, C, D, and G.

mercial suppliers gave reproducible results with only a small range of zone diameters within a particular batch. There was a small variation in the mean zone diameters for discs from different sources of similar stated content although only one batch from each manufacturer was studied.

Discussion

Grouping of beta-haemolytic streptococci by precipitin techniques is time-consuming, and although other serological methods, such as slide agglutination (Rosendal, 1956) and co-agglutination with antibody-coated staphylococci (Christensen *et al.*, 1973; Tebbutt *et al.*, 1976), work well, some laboratories rely on the bacitracin inhibition test to differentiate group A streptococci from strains belonging to the other groups. It is well known that some streptococci of groups B, C, and G can be as sensitive to bacitracin as group A strains, and we have confirmed that the MICs for bacitracin of group A and non-group A streptococci overlap (Fig. 1). The accuracy of the bacitracin inhibition test is therefore partly related to the proportion of these sensitive non-group A strains in the material examined. For instance, in south-east Asia many group C streptococci have zones of inhibition which are similar to those of group A (Maxted, personal communication).

Two methods can be used to interpret the results of the bacitracin inhibition tests; these are either the presence or absence of a zone of inhibition or the choice of a specific zone size as a cut-off point. We have found, using the zone or no-zone criterion, that

the 0.04 unit bacitracin disc was clearly superior to the 0.1 and 0.02 discs using either NCCLS or ACP conditions, although three out of 125 group A streptococci were not identified by the ACP method (Table 2). With the 0.1 unit disc the NCCLS method falsely identified 24 out of 122 non-group A strains as group A, and the ACP method falsely identified 37 of these non-group A strains. Using the 0.02 unit disc, 18 out of 125 group A strains were not identified by the NCCLS method and 27 group A strains were not identified by the ACP method. A specific zone size of 12 mm diameter was chosen for the 0.1 unit disc because it reliably identified group A streptococci using either NCCLS or ACP conditions (Table 3) but the use of a 15 mm zone, as suggested by one manufacturer for this concentration of bacitracin, was not satisfactory. Although Washington *et al.* (1974), using discs containing between 0.02 and 0.04 unit of bacitracin, found that a cut-off point of 11 mm reliably identified group A streptococci, and one manufacturer, using a disc content of 0.04 unit of bacitracin, suggests a zone of 10 mm as a cut-off point, we were unable to find a specific zone size cut-off point with either the 0.02 or 0.04 content disc which reliably differentiated group A from non-group A strains.

Non-group A beta-haemolytic streptococci are being increasingly recognised as human pathogens (eg, Finnegan *et al.*, 1974; Horn *et al.*, 1974) and, whenever possible, identification of these groups should be undertaken. However, group A strains, by virtue of their ability to cause the serious post-infective sequelae of rheumatic fever or glomerulonephritis, remain important and the bacitracin

inhibition test is still the simplest method for their presumptive identification. The findings here show that there is little to choose between a 0·04 unit disc interpreted on a zone or no zone basis and a 0·1 unit disc interpreted by a cut-off point of 12 mm. Since accurate measurement of the 12 mm cut-off point is required the 0·04 unit disc with a zone or no zone reading would appear to be a better choice for routine use.

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