EVALUATION OF A NEW DIFFERENTIAL TEST FOR ENTEROCOCCI IN THE CLINICAL LABORATORY

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STREPTOCOCCI of the Lancefield group D comprise the enterococci (Sherman, 1937), as well as *Streptococcus bovis* and *S. equinus*. Among the former, the species *S. faecalis* (including var. *liquefaciens* and var. *zymogenes*) and *S. faecium* (including var. *durans*) are recognised at present (Deibel, 1964). All enterococci are characterised by growth (a) at both 10°C and 45°C, (b) in media with a pH of 9.6 and in media with a NaCl concentration of 6.5 per cent., and (c) in 0.1 per cent. methylene blue milk with subsequent reduction. They also survive 60°C for 30 min. and form ammonia from peptone (Sherman).

However, no single criterion is specific for enterococci. Growth is observed in *S. bovis*, in group-E streptococci (Breed, Murray and Smith, 1957) and in group-Q streptococci (Nowlan and Deibel, 1967) at 45°C and after 30 min. of exposure to a temperature of 60°C; all group-Q and some *S. bovis* strains (Raj and Colwell, 1966) grow in broth containing 6.5 per cent. NaCl. Group-B streptococci also grow in this medium as well as in Hajna's SF broth (Braunstein, Tucker and Gibson, 1969); and group-N streptococci grow in 0.1 per cent. methylene blue milk (Breed *et al.*). Conversely, occasional enterococcus strains may be atypical in one of these characteristics (Papavassiliou, 1962). Ideally, therefore, a spectrum of tests should be used for the identification of enterococci (Deibel), but this would probably overtax the resources of most clinical laboratories.

In the search for a single convenient test with sufficient clinical reliability, we decided to investigate a report (Booth, 1966) that a disk containing 5 µg of cloxacillin (3-O-chlorophenyl-5-methyl-4-isoxazolyl-penicillin) did not inhibit the growth on Mueller-Hinton agar of any of 25 strains considered to be enterococci; 21 of them had been identified as group D and four were ungroupable, but all hydrolysed aesculin in 40 per cent. bile aesculin agar. In contrast, 17 strains of group-B, one of group-O, and two of ungroupable aesculin-hydrolysing streptococci gave inhibition zones around the disk.

The case for the use of penicillinase-resistant penicillin derivatives for the identification of enterococci is supported by earlier observations that enterococci are resistant *in vitro* to methicillin (dimethoxyphenyl-penicillin), oxacillin (methyl-phenyl-isoxazolyl-penicillin) and dicloxacillin {3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl-penicillin} (Klein and Finland, 1963; Barber and Waterworth, 1964; Hammerstrom *et al.*, 1966). On the other hand, streptococci of the groups A, B, C and E, and viridans streptococci, were found to be sensitive to methicillin and oxacillin (Klein and Finland; Barber and Waterworth; Breitfellner, 1968) as well as to cloxacillin (Jones *et al.*, 1967). The few enterococcus strains reported sensitive to these drugs are either not characterised serologically (Daikos, Kontomichalou and Paradelis, 1963; Fekety and Weiss, 1966), or have later been found resistant (Döll and Brecht, 1968; Duma *et al.*, 1969; and our unpublished observations).

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The small number of streptococci tested against oxacillin and cloxacillin, and the lack of data on the inhibitory action of methicillin disks and on the susceptibility of individual species within the enterococcus group to penicillin derivatives, led us to investigate further the value of such a disk test.

MATERIALS AND METHODS

A total of 320 strains of grouped and ungroupable streptococci was examined (table I). Grouping was done by the precipitation technique (Lancefield, 1933) and group-D species

TABLE I
Results of methicillin and oxacillin disk tests

Lancefield group of streptococci	Number of strains		
	tested	with ≥ 10 mm zone of inhibition around	
		methicillin disk (5 μ g)	oxacillin disk (1 μ g)
A	22	22	22
B	19	19	19
C	20	20	20
D	116	8	9
<i>S. faecalis</i> *	93	0	0
<i>S. faecium</i>	16	4	4
<i>S. faecium</i>	10	0	0
<i>S. faecium</i> var. <i>durans</i>	6	4	4
<i>S. bovis</i>	6	3	4
<i>S. equinus</i>	1	1	1
E	5	5	5
F	4	4	4
G	20	20	20
H	3	3	2
K	3	3	3
L	5	5	5
N	4	4	3
Ungroupable:	99	96	96
α -haemolytic	97	94	94
γ -haemolytic	2	2	2
Total	320	...	...

* Includes *S. faecalis* var. *zymogenes* and var. *liquefaciens*.

were identified biochemically (Deibel); 275 strains had been isolated in our laboratories from human material, and 45 were obtained from other laboratories.

For the disk test, the standard sensitivity method (Bauer *et al.*, 1966) was used, with 6-mm diameter Sensi-Discs (Bio-Quest, Cockeysville, Md.) impregnated with 5 μ g of methicillin or 1 μ g of oxacillin, on Mueller-Hinton Agar (Bio-Quest) containing 4 per cent. sheep blood. Bacteria were considered resistant to methicillin if the diameter of the inhibition zone (including the disk) was less than 10 mm after incubation for 24 hr at 37°C (Bauer *et al.*). Lacking a similar standard value for oxacillin, we assumed that at least the absence of a zone of inhibition indicated resistance.

RESULTS

Whenever inhibition zones were observed, their diameter was 10 mm or more. All strains of groups A, B, C, E, F, G, K and L, two of group H, three of group N, and the two ungroupable γ -haemolytic streptococci yielded zones around both disks (table I); 94 of the 97 ungroupable α -haemolytic streptococci behaved similarly. One strain each of groups H and N was resistant to oxacillin only. Three ungroupable α -haemolytic streptococci were resistant to both methicillin and oxacillin. When tested with a disk containing 10 units of penicillin G these five strains showed zones with diameters larger than 20 mm.

In the enterococcus group, all strains of *S. faecalis* and its varieties were resistant to both drugs (table I). The same was true for the *S. faecium* strains, but four of the six *S. faecium* var. *durans* strains did yield zones. Of the group-D strains other than enterococci, the *S. equinus* strain showed zones, whilst three of the six *S. bovis* strains were resistant to both drugs, and one was resistant to methicillin only.

DISCUSSION

Although a few (3 per cent.) of the ungroupable α -haemolytic streptococci and some *S. bovis* strains showed an aberrant behaviour, the enterococci—and no other streptococci—were almost invariably inhibited by both methicillin and oxacillin. “False-negative” results were seen in *S. bovis* as well as in *S. faecium* var. *durans*. Group-Q streptococci—closely related to group D (Nowlan and Deibel, 1967)—were not available to us. We are unable to explain the three instances of differing results between the methicillin and oxacillin tests, particularly since cross-sensitivity between these drugs—and cloxacillin—has generally been observed in staphylococci, group-A streptococci and pneumococci (Sidell *et al.*, 1963).

On the basis of our findings, a case can be made for the use of the methicillin disk test in the clinical laboratory. Firstly, *S. faecalis* predominates by far among the enterococci from pathological material, whilst *S. faecium* and the var. *durans* are very rare: table II compares the findings of two American and one German group in 1941, 1945 and 1968 respectively. In normal stools, on the other hand, both *S. faecalis* and *S. faecium* can be found (Deibel, 1964). Secondly, traditional tests lack specificity. A recent investigation showed that of 150 group-D streptococci, 100 per cent. hydrolysed aesculin on bile-aesculin medium (BAM), 90 per cent. tolerated heat, 85 per cent. tolerated salt, and 5 per cent. failed to tolerate both heat and salt. Conversely, of 150 α -haemolytic streptococci not groupable in A–O sera, 3 per cent. gave a positive BAM reaction, 17 per cent. were heat tolerant, 10 per cent. were salt tolerant, and 8 per cent. were tolerant to both heat and salt (Facklam and Moody, 1969). Another author found salt tolerance in all of 131 strains of *S. faecium* tested (not including var. *durans*), but only in 86 per cent. of 83 strains of *S. faecalis* (Papavasiliou, 1962). In a further study, 97.8 per cent. of 1792 enterococci and 4.9 per cent. of 2964 other streptococci grew at 45°C (Heeschen, Tölle and Zeidler, 1967). Finally, development of resistance to penicillinase-resistant penicillins among streptococci of groups other than D has not been so far described.

Thus, the test seems both reasonably reliable and convenient, particularly since in many laboratories staphylococci and enterococci are routinely tested against the same array of antibiotics, which includes methicillin.

It should be emphasised that the results of our disk tests cannot for every case be translated directly into in-vivo sensitivity or resistance (Bauer *et al.*, 1966). In this study, we were not concerned with MIC values or with the treatment of streptococcal infections, but rather with a diagnostic test for enterococci in the clinical laboratory.

TABLE II
Distribution of group-D streptococcal species in pathological material from human sources

Reference	Source of cultures	Number of cultures of					Total number of cultures
		<i>Streptococcus faecalis</i>			<i>S. faecium</i> var. <i>durans</i>	unnamed group-D streptococci	
		total	var. <i>liquefaciens</i>	var. <i>zymogenes</i>			
Porch (1941)	genito-urinary tract	73	3	0	0	0	73
Brown and Schaub (1945)	...	339	15	6	1	46	386
Döll and Bredt (1968)	urine	119	37	74	1	0	120

... = Not stated.

SUMMARY

An identification method based on the failure of methicillin (5 µg) and oxacillin (1 µg) disks to inhibit enterococci on Mueller-Hinton Agar was evaluated. All strains tested of *Streptococcus faecalis* and its varieties and of *S. faecium* grew up to the edge of the disks; inhibition of *S. faecium* var. *durans* and of *S. bovis* was variable. In contrast, *S. equinus* and almost all streptococci not belonging to group-D streptococci showed inhibition zones of 10 mm or more.

The methicillin test seems suitable for use in the clinical laboratory in view of the scarcity of *S. faecium* var. *durans*, *S. bovis* and *S. equinus* in pathological material from man and of the margin of error of the traditional tests for enterococci.

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From the American Type Culture Collection, Rockville, Md., we received strains no. 19434 (*S. faecium*), 6056 and 19432 (*S. faecium* var. *durans*), 9809 (*S. bovis*), and 9812 (*S. equinus*).

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