Observations on Anaerobic Glucose Utilization Tests in *Staphylococcus-Micrococcus* Identification

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In comparative tests on 212 freshly isolated and reference cultures of gram-positive cocci, 85 showed anaerobic acid production from glucose in the currently recommended Hugh and Leifson test, but 138 showed anaerobic growth at the expense of glucose in a thioglycolate deep-tube test. Almost all of the discrepant results occurred in strains classified as micrococci of Baird-Parker's subgroups M1 through M4, but of the 106 strains of this type studied by us, only 55 showed anaerobic growth in the thioglycolate test. For practical purposes, the thioglycolate results therefore did not clarify the identification of strains in the M1 through M4 subgroups. Further experiments with modified forms of the pH change and growth detection tests showed that the results of the latter were susceptible to grossly different interpretations by different observers and that equally sensitive but more reliably interpretable results could be obtained with the pH change system.

In recent studies on the microbiology of urethritis in venereal disease clinic patients, we used Baird-Parker's (3) diagnostic scheme to identify the cocci that are very commonly encountered in urethral specimens. The scheme is simple to use in routine situations and allows isolates to be classified as members of the genus *Staphylococcus* or *Micrococcus*, with six subgroups (S1 through S6) in the former and eight (M1 through M8) in the latter. Its use has added a new dimension to studies of the ecology of these bacteria (2), particularly studies of their distribution and potential roles in healthy and diseased body sites (6, 9). In Baird-Parker's scheme, the test used as the sole criterion for allocating a strain to one or other of the two genera is a modified Hugh and Leifson test (7). Strains that produce acid from glucose anaerobically are staphylococci, and those that do not are micrococci. Systematic studies have shown (1) that this method of generic diagnosis conflicts with results of deoxyribonucleic acid (DNA) base composition (mol % guanine plus cytosine [G+C]) studies in that some strains failing to produce acid anaerobically have G+C values similar to those that do and, on the basis of G+C data, could be considered to be staphylococci. It has further been shown that many strains classified as micrococci on their inability to produce acid anaerobically can exhibit some degree of anaerobic metabolism and growth at the expense of glucose (5). These facts, together with

a general recognition that the Hugh and Leifson type of test can occasionally give anomalous results which are difficult to interpret, led Evans and Kloos (4) to devise a simple anaerobic growth test which appears to be more sensitive and produce results that conform with generic diagnoses based on G+C data. Most of the diagnostically aberrant strains in the currently accepted classification (10) are members of Baird-Parker's subgroups M1 through M4, and, since many of our isolates appeared to belong to these groups, we took the opportunity to investigate the relative merits of the Evans and Kloos growth test and the currently recommended Hugh and Leifson test.

MATERIALS AND METHODS

Bacterial strains. We studied 212 strains of gram-positive, catalase-positive cocci, most of which were isolated by us from male genital specimens during 1972.

Methods. The tests described by Baird-Parker (2) were performed on the 212 strains and, in addition, an anaerobic growth test based on the Evans and Kloos (4) method was also conducted. The medium used for this test was Oxoid Thioglycolate medium USP (CM173), with the Ionagar No. 2 (Oxoid) content raised to 0.35%. To facilitate direct comparison, the Hugh and Leifson and thioglycolate tests were conducted as follows. Both media were dispensed in 8-cm columns in tubes (150 by 13 mm) and sterilized. When needed, the media were flash-autoclaved to 121 C, cooled to 55 C, and inoculated while molten with 4

drops of a 24-h-old heart infusion broth (Difco) culture delivered onto the medium surface by a Pasteur pipette. Two tubes of Hugh and Leifson and one of thioglycolate medium were inoculated per strain. Each tube was rotated manually to disseminate the inoculum and cooled. One of the Hugh and Leifson tubes was sealed with sterile liquid paraffin. The results from both cultures were observed after 5 days of incubation at 35 C.

RESULTS

Table 1 shows the classification of the strains based on Baird-Parker's criteria and the comparative results of the Hugh and Leifson and thioglycolate tests. As reported by Evans and Kloos, the majority of discrepant results arose in strains of the M1 through M4 subgroups. Of the 106 strains tested in these groups, 55 gave results in the thioglycolate test which we interpreted as indicating growth anaerobically at the expense of glucose. All gave oxidative (aerobic acid) reactions in the Hugh and Leifson test. Many of these strains showed only weak anaerobic growth in the thioglycolate medium, as described by Evans and Kloos for M1 through M3 strains, and needed very careful comparison with uninoculated controls to discern their positive nature. In view of the fact that we had purposely increased the inoculum size relative to that used by Evans and Kloos in

order to try to minimize this problem, the high incidence of negative and weak reactions in the thioglycolate medium among M1 through M4 strains was disappointing. If the positive thioglycolate results in these strains were accepted as true indicators of their generic affinities, it would mean that 8 of our 15 M1 strains would be reclassified as S4 strains, 20 of our M2 strains would be reclassified as S5 strains, etc. No convenient staphylococcal subgroup exists which could receive the seven apparently aberrant strains of our M4 group. We do not know whether our use of a slightly different thioglycolate medium and tube size for our tests was responsible for the differences between our results and those reported by Evans and Kloos with 14 M1 through M6 strains, but it appears unlikely. Compared with the growth test, the Hugh and Leifson test was simple to interpret, with only three doubtful results in 212 tests. However, in 61 repeat tests, five discrepancies arose in the Hugh and Leifson results, which appears to be an undesirably large number for such a crucial test.

In order to investigate further the question of test sensitivity and the effects of incubation time and operator interpretation upon the results of these two tests, the following experiment was devised.

Bacterial strains. Forty-nine strains were selected from the original 212 to represent subgroups S1 through M7.

Methods. The Hugh and Leifson medium of Baird-Parker (2) was modified by raising the agar concentration to 0.35%. This and the thioglycolate medium (as above) were dispensed in 5-cm columns in tubes (75 by 13 mm). One tube of each medium was used per strain. The tubes were heated and cooled as before, but inoculated heavily with 24-h-old heart infusion broth cultures by stabbing a Pasteur pipette to the tube base and expelling inoculum as withdrawn. After rotation, the tubes were cooled and incubated unsealed at 35 C. They were read at 1-, 2-, and 5-day intervals by four independent operators.

Results of these experiments are shown in Table 2. After 5 days, the S1 through S6 strains yielded 103 out of 104 readings considered positive for anaerobic acid production but only 86 of 104 considered positive for anaerobic growth. In the M5 through M7 strains, the results of the two tests appear very similar, but, in fact, the five anaerobic Hugh and Leifson results were derived from only two strains (four readings from one and one reading from the other), whereas the four thioglycolate anaerobic results were spread between three strains. The M1 through M4 strains still yielded

TABLE 1. Results of Hugh and Leifson and thioglycolate tests on 212 strains of cocci^a

Group or subgroup	No. of strains	Test ^b					
		Hugh and Leifson			Thioglycolate		
		A	A?	O/-	A	A?	O
S1	27	27	—	—	26	—	1
S2	9	9	—	—	8	—	1
S3	1	1	—	—	1	—	—
S4	9	9	—	—	9	—	—
S5	12	11	1	—	12	—	—
S6	27	25	2	—	24	—	3
M1	15	—	—	15	8	—	7
M2	32	—	—	32	20	—	12
M3	44	—	—	44	19	1	23 ^c
M4	15	—	—	15	7	—	8
M5	6	—	—	6	1	—	5
M6	13	—	—	13	2	—	11
M7	2	—	—	2	—	—	1 ^c

^a Strains were classified according to Baird-Parker (3).

^b A, anaerobic glucose utilization; A?, dubious; O, aerobic glucose utilization; O/-, aerobic or negative reaction.

^c One strain was not tested.

TABLE 2. *Effect of incubation time on results (quadruple readings) recorded for 49 strains in the modified Hugh and Leifson and thioglycolate tests*

Group or subgroup	No. of strains	No. of readings	Test ^a											
			Hugh and Leifson						Thioglycolate					
			Day 1		Day 2		Day 5		Day 1		Day 2		Day 5	
			A	O/-	A	O/-	A	O/-	A	O	A	O	A	O
S1-S6	26	104	64	40	94	10	103	1	65	39	78	26	86	18
M1-M4	14	56	2	54	17	39	35	21	8	48	18	38	26	30
M5-M7	9	36	1	35	5	31	5	31	0	36	2	34	4	32

^a A, anaerobic glucose utilization and/or growth; O/-, aerobic or negative glucose utilization; O, aerobic growth only.

TABLE 3. *Fifth-day readings by four operators for 14 strains in subgroups M1 through M4 by using the modified Hugh and Leifson and thioglycolate tests*

Group or subgroup	Strain	Test ^a									
		Hugh and Leifson					Thioglycolate				
		Operator				Original test result ^b	Operator				Original test result ^b
		1	2	3	4		1	2	3	4	
M1	1	A	A	A	A	O	A	A	A	O	O
	2	A	A	A	A	O	O	A	A	A	A
M2	1	A	A	A	A	O	A	A	A	A	A
	2	O	O	O	O	O	O	A	O	O	O
	3	O	O	O	O	O	O	A	O	O	O
	4	A	A	A	A	O	O	A	A	O	O
	5	A	A	A	A	O	O	A	A	O	A
M3	6	A	A	A	A	O	A	A	A	O	A
	1	A	A	A	A	O	O	A	A	O	A
	2	O	O	O	O	O	O	A	A	O	O
	3	A	A	A	A	O	O	A	A	O	O
M4	4	O	O	O	O	O	O	O	O	O	A
	1	O	A	A	A	O	O	O	O	O	O
	2	O	O	O	O	O	O	A	A	O	O
Totals ^c	14	8	9	9	9		3	12	10	1	7

^a A, anaerobic results; O, aerobic result.

^b Refer to data used in Table 1.

^c Except in the first column, these totals refer only to anaerobic readings.

mixed-result patterns in both tests. Overall, the Hugh and Leifson test yielded 143 anaerobic readings, and the thioglycolate test yielded 116 after 5 days.

Table 3 shows the full scoring patterns of the four operators for the 14 M1 through M4 strains after 5 days. Except for one strain, the Hugh and Leifson test was uniformly scored by all four operators, but in the thioglycolate tests only three strains of the 14 were uniformly scored. All of the operators expressed difficulty in deciding how to score the thioglycolate results.

The effect of the operator on the 5-day

readings scored for all 49 strains is shown in Table 4. It further illustrates the disparity between operators in scoring the thioglycolate results compared with their considerable degree of parity in scoring the Hugh and Leifson results.

DISCUSSION

In this study we have tried to assess two simple tests for *Staphylococcus-Micrococcus* differentiation by taking the viewpoint of the non-taxonomist, routine user of such tests.

TABLE 4. *Effect of operator on results recorded on day 5 for 49 strains by using the modified Hugh and Leifson and the thioglycolate tests*

Operator	Test ^a			
	Hugh and Leifson		Thioglycolate	
	A	O/-	A	O
1	34	15	22	27
2	37	12	41	8
3	36	13	36	13
4	36	13	17	32
Original results ^b	26	23	32	17

^a A, anaerobic glucose utilization and/or growth; O/-, aerobic or negative glucose utilization; O, aerobic growth only.

^b Refer to data used in Table 1.

Baird-Parker's diagnostic scheme is a relatively reliable and easily applied tool for routine identification work. At this level of usage, it would be unfortunate if taxonomists allowed their pursuit of natural relationships to dismember Baird-Parker's classification without substantial compensation to its users. Even the most sophisticated classification is a crude reflection of nature. When such a classification is emasculated to produce the kind of diagnostic scheme required in routine bacteriology, it loses most of the philosophical and theoretical niceties of its parental taxonomy and becomes a labeling machine. The particular device under consideration here appears to satisfy the demands of its users. We had no difficulty in correctly classifying known reference cultures included in this study, and the basic principles of the system have been endorsed by the ICSB Subcommittee on the Taxonomy of Staphylococci and Micrococci. (10). In the absence of G+C data, we cannot say whether the results of the thioglycolate test used by us reflect DNA differences between strains or not. The literature suggests that M1 through M4 strains have G+C values in the low thirties, like most S1 through S6 strains. This does not necessarily mean that they are S1 through S6 strains, but it does indicate that they differ from other *Micrococcus* subgroups. None of the anaerobic glucose utilization tests we used appeared to be sufficiently sensitive to serve as a reliable method for differentiating micrococci capable of anaerobic glucose metabolism from those without this capacity, unless of course we accept that strains of both types may occur in the M1 through M4 subgroups. This problem and our observations on the difficulty of

interpreting growth tests may be resolved by technical improvements of the tests. For example, Evans and Kloos did not consider that color-change indicators improved the efficiency of their test, but, for the routine user, such indicators would probably be advantageous. It may also be a fairly simple matter to modify the growth medium to obtain clearer results. We would suggest, however, that before too much taxonomic energy is expended upon such technical problems, the correlation between the metabolic characters and the DNA or genetic characters of these bacteria should be defined more clearly. Although our results with a short-column Hugh and Leifson test agree with the report by Kocur and Mortensen (8) of increased sensitivity, such tests are susceptible to erroneous interpretations resulting from diffusion of acid.

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REPRINT REQUESTS

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