Use of pyruvate fermentation compared with tetrazolium reduction in the differentiation of group D streptococci

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SUMMARY The reduction of 2, 3, 5 triphenyltetrazolium chloride in the original medium of Barnes (*Journal of General Microbiology*, 14, 57, 1956), and in a modified medium, was compared with the ability to ferment pyruvate as a means of separating *Streptococcus faecalis* from all other group D streptococci. The tetrazolium reduction test gave an occasional negative reaction with *Str. faecalis* strains. In addition, a number of strains gave a weakly positive reaction in the test, as did some strains of *Str. faecium* and *Str. bovis*. With some batches of tetrazolium, these weak reactions with *Str. faecalis* were so frequent that interpretation of the results was difficult. On the other hand, all *Str. faecalis* strains, and no other group D streptococci, gave a positive pyruvate-fermentation reaction in 48 hours.

Barnes (1956) showed that *Streptococcus faecalis* could be distinguished from other group D streptococci by its ability to reduce tetrazolium (2, 3, 5 triphenyltetrazolium chloride) to formazan on a glucose-containing agar medium with an initial pH of 6.0. On this medium it forms colonies that have a distinctive red, metallic sheen and are easily distinguished from the colourless colonies of *Str. faecium* and other group D streptococci. It was noted in the laboratory (L. C. Ball, personal communication, 1976) that Barnes' medium failed to support the growth of some strains of *Str. bovis*. The medium was, therefore, modified slightly by substituting Hedley Wright agar (Cowan, 1974) for infusion agar; this gave satisfactory growth of all the group D streptococci tested. Unfortunately batches of tetrazolium varied considerably in their ability to be reduced by *Str. faecalis*, and some strains of *Str. faecium* and *Str. bovis* gave a 'pink-red' coloration of their colonies. These factors tended to make the tetrazolium medium unsatisfactory for distinguishing *Str. faecalis* from other group D streptococci, the main consideration when dealing with clinical material from human sources.

In 1975, Gross *et al.* proposed a test for the fermentation of pyruvate as a means of distinguishing *Str. faecalis* from both *Str. faecium* and *Str.*

bovis. The pyruvate-fermentation test was therefore compared with tetrazolium reduction as a means of distinguishing *Str. faecalis* from other group D streptococci.

Media

HORSE BLOOD AGAR

A layer of Hartley digest agar containing defibrinated horse blood 5% v/v (Oxoid) was poured over a layer of peptone-water agar (Cowan, 1974).

TODD-HEWITT BROTH

This was prepared as described by Cowan (1974).

INFUSION AGAR. pH 6.0

0.455 kg of minced fat-free meat; M Peptone No. 1 (Lab. M), 10 g; sodium chloride, 5.0 g; Davies agar, 10 g; and 1 litre of cold tap water.

Minced fat-free meat was placed in the water to infuse overnight; the next morning the rest of the ingredients were added, and the mixture was boiled for 30 minutes, then filtered to remove the meat, which was discarded. The pH was adjusted to 6.0 and the medium was autoclaved for 20 minutes at 115°C.

TETRAZOLIUM MEDIUM (Barnes, 1956)

This was infusion agar, pH 6.0, with the addition of

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glucose 1% w/v and 2, 3, 5 triphenyltetrazolium chloride 0.02% w/v (Koch-Light Laboratories, Ltd).

Five batches of tetrazolium, nos. 40761, 44448, 55750, 69128, and 72919, were used.

HEDLEY WRIGHT AGAR, pH 6.0

This was prepared as described by Cowan (1974); the final pH was adjusted to 6.0.

MODIFIED BARNES' TETRAZOLIUM MEDIUM

This was Hedley Wright agar, pH 6.0, with the addition of glucose 1% w/v and 2, 3, 5 triphenyltetrazolium chloride.

PYRUVATE MEDIUM

This contained Difco Tryptone, 10 g; Difco Yeast Extract, 5 g; potassium dihydrogen phosphate, 5 g; sodium chloride, 5 g; sodium pyruvate, 10 g; bromothymol blue, 0.04 g, and Davis Agar, 10 g; distilled water, 1 litre.

This mixture was boiled for 5 minutes to dissolve the constituents completely, and the pH was adjusted to 7.2-7.4. The medium was dispensed and bottled into 100 ml amounts, and sterilised by autoclaving at 120°C for 15 minutes. When plates were required, the bottled medium was melted and poured; these poured plates could be stored at 4°C for at least four weeks. The medium was blue-green in colour.

Cultures

The group D streptococcal strains examined were: (1) from the National Collection of Type Cultures: *Str. faecalis*, nos. 2705, 5957, and 775; *Str. faecium*, nos. 7171, 7176, 7182, 7379, 7380, and 7174; *Str. durans*, nos. 8129, 8130, 8174, and 8307; *Str. bovis*, no. 8140; and (2) a collection of 99 strains of *Str. faecalis*, 26 strains of *Str. faecium*, 1 strain of *Str. durans*, and 20 strains of *Str. bovis* received for identification in the laboratory and tested by the methods described by Parker and Ball (1976).

Methods

CULTIVATION OF TEST ORGANISMS

Both routine and NCTC strains were plated for purity on horse-blood agar and incubated overnight at 37°C in an atmosphere of 5% CO₂. A well isolated colony was subcultured into 10 ml of Todd-Hewitt broth, which was incubated overnight at 37°C in 5% CO₂. Next morning a drop of turbid broth was dropped on to freshly poured pyruvate and tetrazolium plates, which were incubated aerobically at 37°C for two days. Controls were

included: *Str. faecalis* no. NCTC2705, which gave strongly positive results in both tests, and *Str. faecium* no. NCTC7171, which gave negative results in both tests.

RECORDING OF RESULTS

Reduction of tetrazolium was graded as follows: +++ indicated the classical appearance of red colonies with a metallic sheen; ++, deep pink colonies without the metallic sheen; +, faintly pink colonies; and -, no coloration of the colonies.

In the pyruvate-fermentation test a bright yellow coloration of the colonies and surrounding medium indicated acidification and was a positive result; no change in colour was a negative result. Weak reactions after 48 hours never occurred.

Results

Table 1 illustrates the variation of tetrazolium reduction by 102 strains of *Str. faecalis* (all of which survived 60°C for 30 minutes, failed to acidify arabinose, and possessed Lancefield group D antigen) with different batches of the chemical. It also compares the effect of different basal media, both Barnes' and the modified Hedley Wright agar, on the action of tetrazolium. In this series of tests the intensity of reduction was in general greater on the modified medium than on the original medium, but the latter gave slightly fewer completely negative 'false' reactions. The performance of both media was greatly influenced by the batch of 2, 3, 5 triphenyltetrazolium chloride used. For example, in the modified media, batch no. 69128 gave good reduction with 90/102 *Str. faecalis* strains, and a further eight might have been accepted as giving a positive reaction (+++); only four of 102 organisms tested gave very feeble reactions (+) that were potentially misleading, and none gave completely negative reactions. On the other hand, batch no. 72919 performed very poorly in both types of media and would have led to a great deal of difficulty in the interpretation of the results by inexperienced observers. Less than one-third of all the cultures gave strongly positive results (+++) and nearly one-quarter gave equivocal results (+). The other batches of tetrazolium tested, nos. 40761, 44448, and 55750, all performed moderately well, but a number of *Str. faecalis* strains gave equivocal or 'false' negative results on both types of medium.

Table 2 presents the frequency with which group D streptococci gave positive reactions of any strength from + to +++. The strains tested were representative of the more commonly isolated species: *Str. faecalis*, *Str. bovis* biotype I (see Parker and Ball, 1976), *Str. faecium*, and *Str. durans*. Two

Table 1 Variation of tetrazolium reduction by *Str. faecalis* with different batches of 2, 3, 5 triphenyltetrazolium chloride in modified Barnes' medium (MBM) and original Barnes' medium (BM)

Degree of reduction	Number of cultures (of 102 tested) that gave the indicated degree of reduction on medium containing tetrazolium batch number									
	40761 MBM	BM	44448 MBM	BM	55750 MBM	BM	69128 MBM	BM	72919 MBM	BM
+++	68	55	61	70	70	56	90	80	32	29
++	19	40	30	16	13	35	8	15	40	45
+	13	5	11	14	15	10	4	7	22	24
-	2	2	0	2	4	1	0	0	8	4

+++ red colonies with metallic sheen

++ deep pink colonies

+ faintly rose-pink colonies

- no pink or red coloration

MBM = modified Barnes' medium

BM = original Barnes' medium (see text)

Table 2 Frequency of positive tetrazolium reduction tests (of any strength from + to +++) and of positive pyruvate-fermentation tests given by strains of various group D streptococci

Streptococcus	No. of cultures tested	Number of cultures of the indicated streptococci that gave a positive reaction on medium containing:											
		Tetrazolium, batch no.										Number of cultures fermenting pyruvate	
		40761 MBM		44448 MBM		55750 MBM		69128 MBM		72919 MBM			
		B		B		B		B		B		B	
<i>faecalis</i>	102	100	100	102	100	98	101	102	102	94	98	102	
<i>faecium</i>	30	2	2	2	2	2	3	2	2	2	2	0	
<i>bovis</i> biotype I	21	1	1	1	1	1	1	1	1	1	1	0	
<i>durans</i>	5	0	0	0	0	0	0	0	0	0	0	0	

strains of *Str. faecium* (no. NCTC7182 and one routine isolate) and one of *Str. bovis* biotype I gave 'false' positive reactions indicated by pink or red colonies. Indeed, *Str. faecium* no. NCTC7182 gave a ++ reaction and would certainly have been misclassified as a *Str. faecalis* if tetrazolium reduction had been used as the identification criterion.

The variation of the basal medium had little effect on the frequency of 'false' positives. On the other hand, all *Str. faecalis* strains and none of other group D streptococci tested gave a positive pyruvate-fermentation test.

Discussion

The group D streptococci of importance in medical microbiology are *Str. faecalis* and *Str. bovis*. These are unlikely to be confused, because *Str. bovis* usually forms minute colonies and is highly sensitive to benzyl penicillin in a disc-diffusion test. The main problem, therefore, is to separate *Str. faecalis* from the rest of the enterococci (especially *Str. faecium* and *Str. durans*), and the tellurite resistance or the tetrazolium reduction tests are usually used for this purpose in the routine lab-

oratory. In our earlier experience, the latter gave a more clear-cut differentiation than the former, and this test plus the acidification of arabinose, melibiose, and melezilose greatly facilitated presumptive identification of these group D streptococci. However, we were from time to time troubled by batches of 2, 3, 5 triphenyltetrazolium chloride that gave a number of feeble or even negative reactions with strains of streptococci that appeared to be in every respect true *Str. faecalis*.

Gross *et al.* (1975) proposed an alternative reaction which utilised the ability of *Str. faecalis* to ferment pyruvate. These authors found that this metabolic ability was exclusive to *Str. faecalis* among the group D streptococci. They did observe, however, that some strains of *Str. avium* gave a feeble positive reaction after 72 hours, but these same strains were negative if incubation was for only 48 hours. They did not test any group D streptococci that gave equivocal results with tetrazolium reduction, but in this study I included a number of 'problem' strains. From the results presented I conclude that pyruvate-fermentation is an excellent key test to use in the differentiation of *Str. faecalis* from other group D streptococci.

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