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 triphenyl-
tetrazolium 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 
auctoclaving 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 triphenyl-
tetrazolium chloride used. For example, in the 
modified media, batch no. 69128 gave good reduc-
tion 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 interpre-
tation 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
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 laboratory. 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 meleziloze 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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References


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