

THE USE OF A BILE-AESCULIN MEDIUM AND OF MAXTED'S TECHNIQUE OF LANCEFIELD GROUPING IN THE IDENTIFICATION OF ENTEROCOCCI (GROUP D STREPTOCOCCI)

BY

ALEXANDER SWAN

From the Department of Pathology, King's College Hospital Medical School, University of London

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The name enterococcus, originally given by Thiercelin (1899) to a Gram-positive coccus of faecal origin growing in pairs and short chains, has been widely used for a group of morphologically similar streptococci found in the gut of man and other mammals, in dairy products, and in soil. The limits of this group, formerly rather vague, were satisfactorily defined with the application to it of Lancefield grouping methods by Sherman and his collaborators (Sherman, 1937; Sherman, Mauer, and Stark, 1937; Sherman, Stark, and Mauer, 1937; Sherman, Stark, and Yawger, 1937), and later by Shattock and Mattick (1943) and Shattock (1945 and 1949b). From this work it is apparent that the great majority, if not all, enterococci belong to Lancefield Group *D*, and that in fact this serological property should be taken as the basis of their classification. The group will then include the following species: *Strep. faecalis*, with its varieties, *zymogenes*, and *liquefaciens*, *Strep. durans*, and *Strep. bovis*.

The degree of variation in the biological properties of Group *D* streptococci is well shown in Table I reproduced from Shattock (1949b; see also Nyman, 1949).

Table I covers the majority of important biological properties of the group, except bile tolerance, or the ability to grow in the presence of bile in concentrations up to 40% (Weissenbach, 1918), which is usually regarded as common to all species of enterococci, and aesculin hydrolysis.

Attention was drawn to the value of aesculin hydrolysis in the identification of enterococci by Rochaix (1924), and the use of aesculin in bile media was regarded as the best single differential test by Meyer and Schönfeld (1926), who found that 61 of their 62 strains of enterococci hydrolysed aesculin, as compared with only six among

82 strains of *Strep. viridans*. Weatherall and Dible (1929), who obtained similar results, 60 of their 61 bile-tolerant, heat-resistant, mannitol-fermenting diplococci hydrolysing aesculin, considered, however, that the hydrolysis of aesculin in a bile medium was "very little more than an indicator" of bile tolerance. This conclusion was probably responsible for the fact that the test has been little used. The work of Williams and Hirch (1950) suggests, however, that aesculin hydrolysis has an independent value in the classification of bile-tolerant cocci, as only 83.6% of their 140 strains of bile-tolerant streptococci hydrolysed aesculin.

The present paper records an attempt to find a simple, rapid and accurate single procedure for the recognition of enterococci as a group, minor subdivisions of which are of little importance in the routine diagnostic work of a medical laboratory. Results of Lancefield *D* grouping were compared with those of tests for bile tolerance and aesculin hydrolysis, as well as heat resistance, on a number of freshly isolated and stock strains of streptococci.

Source of Material

One hundred and forty-two strains of streptococci and eight strains of micrococci were isolated from human material in the laboratory of a general hospital. The sources of these strains are shown in Table II.

In addition, nine stock strains of *Strep. bovis*, three isolated from human intestine and six from the rumen of the cow, and 12 strains of *Strep. durans* were examined. Of the latter, two were recovered from the human intestines, the remainder being mostly obtained from milk and dairy products.

TABLE I
DIFFERENTIATION OF SPECIES WITHIN GROUP D REPRODUCED FROM SHATTOCK (1949a)

Species	Haemolysis	Gelatin Liquefaction	Strong Reduction of Litmus Milk	Mannitol	Sucrose	Raffinose	Survival at 60° C. for 30 min.	Growth	
								at 45° C.	At pH=9.6
<i>Strep. faecalis</i> ..	—	—	+	+	±	—	+	+	+
<i>Strep. faecalis</i> , variety <i>zymogenes</i> ..	+	±	±	+	+	—	+	+	+
<i>Strep. faecalis</i> , variety <i>liquefaciens</i> ..	—	+	+	+	+	—	+	+	+
<i>Strep. durans</i> ..	±	—	—	—	—	—	+	±	±
„ <i>bovis</i> ..	—	—	—	±	+	+	—	+	—

± = Varies from strain to strain.

TABLE II
SOURCES OF STRAINS ISOLATED IN THE HOSPITAL LABORATORY

Sources	Presumptive Enterococci	Presumptive <i>Strep. viridans</i>	Micrococci	Total Numbers of Strains
Faeces ..	65	1	8	74
Urine ..	38	0	0	38
Vagina ..	10	0	0	10
Wounds, abscesses, etc.	5	0	0	5
Blood cultures	2	0	0	2
Sputum ..	1	0	0	1
Throat ..	0	20	0	20
Total ..	121	21	8	150

Methods of Isolation

One hundred and thirty strains of Gram-positive cocci were selected on the basis of their colonial morphology on McConkey medium. They appeared as small, discrete colonies, most of which were lactose fermenting. Microscopical examination of broth cultures showed eight of these strains to be micrococci and one to be a long-chained streptococcus. The remaining 121 strains, which grew in pairs or short chains, have, for convenience, been referred to throughout this paper as “presumptive enterococci.”

To contrast with these, 20 strains of alpha-haemolytic streptococci were isolated from the throats of healthy persons. These strains have been classed with the alpha-haemolytic long-chained streptococcus of faecal origin as “presumptive *Strep. viridans*.”

Methods of Testing

All strains were examined by the following tests: Lancefield grouping, heat-resistance, bile-tolerance, aesculin-hydrolysis.

Sixty-eight strains were also tested for fermentation of mannitol and the ability to grow in nutrient broth containing 6.5% NaCl, on blood-agar containing 0.06% potassium tellurite, and in milk containing 0.1% methylene blue, with the decolorization of the dye.

Lancefield Grouping.—(1) The hot-HCl-hydrolysis technique of Lancefield (1933) was employed throughout, using the sediment from 10 ml. of overnight glucose-broth cultures.

(2) All freshly isolated strains (150) were moreover grouped by a modification of the enzyme method of Maxted (1948). The enzyme was prepared by freezing and thawing an agar culture of *Streptomyces albus*. The fluid collected after thawing, which contains the enzyme, was stored without preservative at a temperature below 5° C.; under these conditions its activity was retained for some months. Seitz filtration and pH adjustment were omitted on Mr. W. R. Maxted's advice. The streptococci were grown in 10 ml. of glucose broth, or beef-digest broth, for 18 hours. After centrifuging, the supernatant fluid was discarded, and the deposit placed for two minutes in boiling water. After cooling, 0.5 ml. of the proteolytic extract was added and the mixture incubated at 50° C. overnight. As previously noted by Maxted (1948), Group D streptococci were found rather resistant to the action of the enzyme, and even after this prolonged incubation the lysis was usually incomplete, making recentrifugation necessary. Satisfactory bacterial extracts were also obtained from enzyme-treated saline suspensions of growth on blood-agar plates.

Streptococcal extracts prepared by both Lancefield's and Maxted's methods were tested in the same way by layering on to grouping serum (obtained from Messrs. Burroughs Wellcome, Ltd.) in glass tubes of 1 mm. internal diameter. The results were read during the following 30 minutes.

Heat-resistance Test.—Approximately 1 ml. of an 18 to 24 hours' broth culture was placed in a 60° C. water-bath; a loopful was removed for subculture on blood-agar after 15, 30, 45, and 60 minutes. Strains surviving this temperature for 30 minutes were termed heat-resistant (Orla-Jensen, 1919; Dible, 1921).

Bile-tolerance Test.—Heavy inocula were made on segments of a nutrient-agar plate containing 5% horse serum, 0.1% aesculin, 0.05% ferric citrate, and sufficient “difco” dehydrated ox-gall powder to give a final concentration corresponding to 40% bile (Williams and Hirch, 1950). The plates were examined for growth after 24 hours' incubation.

Hydrolysis of Aesculin.—This property was tested on the previously described bile-aesculin plate. Strains which hydrolysed aesculin produced a black colour in and immediately around the colonies. Bile-tolerant organisms which did not hydrolyse aesculin gave rise to whitish colonies. The plates were incubated for 24 hours; growth for a longer period was found not only unnecessary but also undesirable as it was liable to obscure the results, when as many as 12 strains were cultured on one plate. As Weatherall and Dible (1929) have pointed out, this is not truly a test of the capacity of an organism to hydrolyse aesculin, since strains which are not bile-tolerant do not grow on this medium and are, therefore, not tested.

Results

The results are summarized in Tables III and IV.

Of the 121 "presumptive enterococci," 117 belonged to Lancefield Group *D*. All these Group *D* strains were heat-resistant, bile-tolerant, and all hydrolysed aesculin. Of the four strains which did not react with Group *D* serum, two were heat-resistant but intolerant to bile, and two were neither heat-resistant nor bile-tolerant. These four strains were alpha-haemolytic.

None of the 21 strains of "presumptive *Strep. viridans*" reacted with Group *D* serum and 19

of them were neither heat-resistant nor bile-tolerant; two strains grew on bile-aesculin plates without hydrolysing aesculin, but neither was heat-resistant.

None of eight strains of micrococci was heat-resistant and none hydrolysed aesculin, but six were bile-tolerant.

All *Strep. bovis* and *Strep. durans* strains were bile-tolerant and hydrolysed aesculin, their extracts reacted with Group *D* serum without concentration. All strains of *Strep. durans* were, moreover, heat-resistant, but only four out of the nine strains of *Strep. bovis* survived 60° C. for 30 minutes.

Table IV shows the results of the subsidiary four tests, mannitol fermentation and growth in media containing 6.5% NaCl, or 0.06% potassium tellurite, and in milk containing 0.1% methylene blue, performed on 68 of the above 117 Group *D*, heat-resistant, bile-tolerant, aesculin hydrolysing strains. Only 60 (88.3%) strains gave positive results with all four tests.

All cocci (150 strains), with the exception of stock strains of *Strep. bovis* and *Strep. durans*, were grouped by both Maxted's and Lancefield's methods. Both techniques gave identical precipitation results with Group *D* sera in all cases except one; this strain gave a positive Group *D* reaction on acid extraction but consistently negative results when extracted by Maxted's method.

TABLE III
RESULTS OF TESTS ON STRAINS OF ENTEROCOCCI

Total No. of Strains Tested	No. of Strains with Common Properties	Lancefield Group <i>D</i>	Heat Resistance	Bile Tolerance	Aesculin Hydrolysis
Presumptive enterococci 121	117	+	+	+	+
	2	—	+	—	—
Micrococci 8	6	—	—	—	—
	2	—	—	+	—
Presumptive <i>Strep. viridans</i> 21	2	—	—	—	—
	19	—	—	+	—
<i>Strep. bovis</i> 9	4	+	+	+	+
	5	+	—	+	+
<i>Strep. durans</i> 12	12	+	+	+	+

TABLE IV
RESULTS OF ADDITIONAL TESTS ON 68 STRAINS OF ENTEROCOCCI

Numbers and percentage of strains	Positive	Growth in Medium Containing 6.5% NaCl	Fermentation of Mannitol	Growth on Plate Containing 0.06% Pot. Tellurite	Growth in Medium Containing 0.1% Methylene Blue
		66 (97%)	67 (98.5%)	64 (94%)	63 (93%)
	Negative	2	1	4	5

Discussion

All 138 Group *D* strains, in this survey, and none outside this group were found to possess the property of aesculin hydrolysis. Bile tolerance, although equally constant within the group, was shared by some (six out of 33) strains outside it, and is therefore less specific for enterococci. Heat resistance is evidently neither a constant nor exclusive character of Group *D* streptococci, for five out of nine strains of *Strep. bovis* failed to survive 60° C. for 30 minutes, and on the other hand two of the 25 strains of *Strep. viridans* (alpha-haemolytic streptococci not belonging to Group *D*) proved to be heat-resistant.

The behaviour of two strains of alpha-haemolytic, non-Group-*D* streptococci and of six strains of micrococci, which grew on the bile aesculin plate without hydrolysing aesculin, together with the previously quoted figures from Williams and Hirch (1950), point to the value of including aesculin in a bile medium.

The bile-aesculin plate, therefore, appears to be a simple reliable means for the recognition of enterococci. It is much simpler and cheaper than

Lancefield grouping, since as many as 12 strains can be inoculated on one plate.

The following procedure has been adopted in the routine work of this laboratory for the presumptive identification of enterococci. The Gram-positive cocci are subcultured (*a*) in 10 ml. of glucose broth and (*b*) on a bile-aesculin plate. The next day the morphology of the growth in glucose broth is examined in a hanging drop. Strains growing in pairs and/or short chains and hydrolysing aesculin are classified as enterococci. The glucose broth culture is used only for the grouping of those strains which fail to hydrolyse aesculin.

It must be stressed that in this preliminary survey very small numbers of streptococci other than those of Group *D* have been examined. Thus, whereas the property of aesculin hydrolysis in a bile-containing medium has been satisfactorily demonstrated among enterococci, its specificity for the group remains to be proved.

Summary and Conclusions

In an attempt to select a single reliable and simple method for the identification of enterococci (Group *D* streptococci) the following principal tests were employed: Lancefield grouping, heat-resistance, bile-tolerance, and aesculin hydrolysis. One hundred and seventeen Lancefield Group *D* strains were found to be heat-resistant, bile-tolerant, and aesculin-hydrolysing. Of 25 alpha-haemolytic streptococci, not of Group *D*, none hydrolysed aesculin, but two were heat-resistant and two were bile-tolerant. From the very high correlation between Lancefield grouping and hydrolysis of

aesculin on a bile-containing medium it has been concluded that the latter is a valid test for the recognition of enterococci, having the advantage of simplicity over the former.

Extracts for Lancefield grouping were prepared by two methods: (1) the hot-HCl technique of Lancefield and (2) the enzymic method of Maxted. Both methods gave equally good results.

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