DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND OF DAIRY ORIGIN BY METHYLENE BLUE TOLERANCE AND FINAL ACIDITY

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Although hemolysis on the blood agar plate was formerly considered a property peculiar to streptococci from pathogenic sources, it is now recognized that the same property is also possessed by many non-pathogenic strains. Means of distinguishing hemolytic strains that are pathogenic from those that are non-pathogenic is especially important in the sanitary examination of dairy products. Previous studies (1–9) on streptococci have dealt with the properties of hemolysis, final acidity, and reduction of dyes, but the correlation of the results has been made difficult by the fact that the different workers have had different interests. The medical bacteriologist has usually studied strains isolated from infections and has emphasized hemolysis or acid production without regard to dye reduction. The agricultural bacteriologist on the other hand has studied non-pathogenic or saprophytic strains isolated from milk products and has emphasized acid production or dye reduction without regard to hemolysis.

The present investigation was planned to attempt to correlate these differential characters previously used in their respective fields by medical and agricultural bacteriologists. In spite of the fact that no new methods were introduced, the results are of new interest: (1) the collection of hemolytic streptococci included both pathogenic strains representative of those associated with human disease and saprophytic strains representative of the normal flora of milk products; (2) the three tests (hemolysis, final acidity, and dye reduction) are applied at the same time to strains from the different sources.

EXPERIMENTAL

Final Acidity and Source of Strains. The 138 strains of hemolytic streptococci included in our tests may be separated on the basis of their final hydrogen ion concentration into the two main groups described by Ayers (1) and by Avery and Cullen (2): those having a final acidity range of pH 5.3 to 5.0 represent the low acid group; the others with a range of pH 4.5 to 4.0, the high acid group.

TABLE I

Hemolytic Streptococci

Source	Number of strains
Low acid producing strains	
Sputum, pneumonia	6
Blood, septicemia, meningitis, osteomyelitis	7
Pleural fluid, empyema	19
Lung, pneumonia	12
Throats, pneumonia, measles	43
Pus, abscesses, cellulitis, scarlet fever	4
Cows' udders	4
Total	95
High acid producing strains	
Cheese	21
Butter starter	1
Milk (pasteurized 1, certified 2)	6
Udders	14
Cow feces	1
Total	43

Table I summarizes the grouping of the strains on the basis of final acidity of glucose broth cultures. All the strains from human sources fall in the group of low acid producers. While 4 of the strains included in the low acid producing group were isolated from the udders of cows, two of them were identified as belonging to the human type and were known to be responsible for two separate epidemics of septic sore throat. Hence, all but two of the 95 strains in the low acid

producing group were definitely associated with human infection. In contrast to this, all of the 43 strains producing high acidity had been isolated either from bovine sources or from dairy products.

Methylene Blue Reduction

All strains both of the low and high acid producing groups were tested for their capacity to reduce methylene blue in milk.

Three different concentrations of the dye were used: 1 to 20,000, 1 to 10,000 and 1 to 5,000. Methylene blue milk was prepared by adding a sterile 0.4 per cent aqueous solution of medicinal methylene blue (Merck's) to sterile fat-free milk in amounts sufficient to give the desired final concentration. Three tubes of methylene blue milk, containing respectively 1 to 20,000, 1 to 10,000 and 1 to 5,000 concentrations of the dye, were inoculated with 0.1 cc. of 24 hour broth culture of

TABLE II

Reduction of 1: 5000 Concentration of Methylene Blue in Milk by High and Low Acid Producing Strains

Final acidity	Methylene Blue Reduction	
	Strains not reducing	Strains reducing
Low acid producing group	94	1
High acid producing group	19	24

the strain to be tested. The inoculated tubes were incubated at 37°C. and observed for dye reduction over a period of seven days.

The tests with 1 to 5,000 concentration of methylene blue furnished the most definite distinction. As shown in the summary (Table II), 94 of the 95 low acid producing strains failed to reduce methylene blue. (Ninety-one of these low acid producing and non-dye reducing strains were isolated from human source). On the other hand, the high acid producing strains, all of which were isolated from dairy sources, can be separated into two groups; strains which reduced and those which did not reduce the dye. It is important that 14 of the 19 high acid producing strains that failed to reduce the methylene blue had been isolated from udders of cows; and that 21 of the 24 high acid producing strains that reduced the dye were from various kinds of cheese.

Bactericidal Action of Methylene Blue

Negative subcultures were obtained in tests of viability of the streptococci introduced as the test inoculum in the cultures in which no reduction of the dye had occurred. The apparent absence of living streptococci indicated that failure to reduce the dye in the preceding tests represented a lack of tolerance to methylene blue on the part of the non-reducing strains. Apparently in the unreduced state, the methylene blue either prevented the growth of the non-reducing strains (bacteriostatic action) or destroyed their life (bactericidal action).

In order to obtain further information on the influence of methylene blue upon the viability of the streptococci a constant amount (0.1 cc.) of broth cultures of reducing and of non-reducing strains was inoculated into milk containing a known concentration of the dye; plate cultures were made after different intervals of time. The original number of streptococci added to the methylene blue milk was known to be quantitatively sufficient to establish the growth of the more delicately growing strains in the medium containing no methylene blue.

The results of the typical experiments summarized in Table III show that approximately 90 to 97 per cent of the non-reducing streptococci were killed by 10 minutes exposure to a 1:5000 concentration of methylene blue in milk. Different strains of the non-reducing group varied somewhat in the time required to kill 100 per cent of the cells; with some strains, complete sterility was obtained within 30 to 60 minutes but with others a few viable cells persisted for several hours; in all cases, the cultures were completely sterile within 48 hours.

DISCUSSION

A collection of hemolytic streptococci from both human and dairy sources was studied in order to determine if the combined use of the properties of dye reduction and of final acidity would be of value in the differentiation of hemolytic strains of diverse origins.

Different concentrations of methylene blue were tried but it was found that a solution of 1 to 5,000 in milk gave the most clear cut separation of the strains into dye-sensitive and dye-tolerant groups. This concentration of the dye not only prevented growth but was

actually bactericidal to the dye-sensitive strains. The dye-tolerant strains grew in the presence of and reduced a 1:5000 concentration of methylene blue. Of the 113 dye-sensitive strains, 108 were isolated from human or bovine sources and in many instances were associated with infectious processes in man or cattle. The other five strains were from samples of fresh and certified milk and were probably of udder origin (hemolytic udder streptococci are more commonly found in freshly drawn and certified milk than in ordinary market milk where the more resistant varieties of milk streptococci predominate (7, 9)). In contrast to the direct animal origin of the dye-sensitive hemolytic

TABLE III

Hemolytic Streptococci; Bactericidal Action of 1:5000 Methylene Blue Milk

Period of Exposure to Methylene Blue 1:5,000	Number of viable organisms per cmm.			
	Strains not reducing Methylene Blue		Strains reducing Methylene Blue	
	Strain "2"	Strain "4"	Strain "P"	Strain "M"
minutes				
0	2,200	3,500	2,500	3,000
5	460	770	2,400	2,700
10	40	320	2,000	2,800
15	9	300	3,000	3,600
20	1	210	4,500	6,000
30	0	60	6,000	7,100
60	0	25	11,000	13,000

streptococci, the 25 dye-tolerant strains with only two exceptions[1] were isolated from pasteurized milk, cheese and commercial butter starter.

When both the methylene blue and the final acidity tests are applied to hemolytic streptococci from human and dairy sources, three groups are distinguished. First, a group of low-acid-producing strains that are sensitive to methylene blue; this corresponds to Avery and Cullen's low-acid-group from human sources. Second, a group of high-acid-

[1] Although one of these strains was isolated from a human throat and the other from a cow's udder, their presence in these locations might have been due to their introduction from saprophytic sources; e.g., recently ingested food in the case of the throat strain, or the cow's bedding in the case of the udder strain.

producing strains that are sensitive to methylene blue, these are most commonly derived from the normal or diseased udder. Third, a group of high-acid-producing strains that are tolerant to methylene blue. This last group, the dye-tolerant and high-acid-producing hemolytic streptococci are different from the high-acid-producing but dye-sensitive strains included in Avery and Cullen's group of "bovine" strains. The latter are most frequently of udder origin and are often associated with bovine mastitis; their presence in dairy products is for the most part limited to fresh raw milk for they are relatively infrequent in older market milk or in stored dairy products like butter or cheese. The dye-tolerant group on the other hand are in general much more resistant to unfavorable conditions than are most hemolytic streptococci and consequently are found in cheese or other dairy products that have been subjected to storage or other processes which tend to kill off the dye-sensitive strains.

The grouping of streptococci upon this basis, while not an attempt at a systematic classification of the *Streptococcus* genus, is of interest from two different points of view. The results as a whole indicate, that with hemolytic streptococci there is an apparent relation between lack of tolerance to methylene blue and tendency toward parasitism; and that all three tests, namely hemolysis, final pH, and methylene blue reduction should be applied in the study of streptococci of disputed etiological and epidemiological significance.

SUMMARY

A grouping of 138 strains of hemolytic streptococci based on differences in dye-sensitiveness and in final hydrogen-ion concentration of cultures is presented. Three groups are distinguished; (1) human parasitic strains, defined by a final pH range of 5.2 to 5.0 and by failure to reduce methylene blue (1:5000) in milk, (2) bovine strains parasitic in the udder, characterized by a final pH range of 4.5 to 4.2 and by failure to reduce methylene blue (1:5000) in milk, (3) saprophytic strains, characterized by a final pH range of 4.5 to 4.2 and by ability to reduce methylene blue.

Methylene blue was bactericidal for the strains of hemolytic streptococci that fail to reduce it, but neither bacteriostatic nor bactericidal for the strains that caused its reduction.

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