STUDIES UPON MINUTE HEMOLYTIC STREPTOCOCCI

I. The Isolation and Cultural Characteristics of Minute Beta Hemolytic Streptococci*

By PERRIN H. LONG, M.D., and ELEANOR A. BLISS, Sc.D.

(*From the Department of Medicine, The Johns Hopkins University, Baltimore*)

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In a recent brief report (1) we discussed the isolation of small, amphophilic cocci which possess the power of producing the *beta* type of hemolysis in poured plates of rabbit's blood, sugar-free agar. These minute organisms resembled the ordinary type of *beta* hemolytic streptococci in many of their morphological and cultural characteristics. They were isolated from the rhinopharynx in a variety of diseases and from normal human beings, but were recovered most frequently from the throats of individuals suffering from glomerular nephritis or progressive rheumatic infection. These organisms occur in pairs, short chains and masses and are one-half to two-thirds the size of ordinary *beta* hemolytic streptococci. While it seems unlikely that these organisms have not been previously observed, a thorough search of the available literature has failed to reveal any description of them. In this report their cultural characteristics will be described.

Methods

Technique of Primary Isolation.—The majority of our strains have been isolated from the throats of human beings and we believe that the method of swabbing the throat is of prime importance. We have found that cotton-tipped sterile twisted wire swabs are ideal for this purpose. They must not be tipped with too much cotton. The ordinary cotton-tipped, wooden applicators which one generally encounters on hospital wards are too bulky and too difficult to maneuver in the process of swabbing a throat. It is our practice to rub thoroughly each tonsil or tonsillar fossa, exploring the tonsillar crypts, if any are present, and

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then to swab the tissues behind the pillars and on the posterior pharyngeal wall. Two of the swabs held together are used in this procedure.

The swabs are transferred to small test tubes containing 1 cc. of sterile physiological saline and are vigorously shaken in the salt solution. One 4 mm. loopful of this suspension is inoculated into a tube containing 12 cc. of melted 2 per cent, sugar-free, beef infusion agar (temperature 45°C.), 1 cc. of sterile defibrinated rabbit's blood is added and the whole is thoroughly mixed and poured into a sterile Petri dish. The following formula is used in preparing the agar. To 1 pound of freshly ground, lean, raw beef is added 1 liter of tap water and the mixture is infused overnight in the ice box. On the following day the fat is removed and the residue is heated to between 85–90°C. for 30 minutes and is filtered through grey French filter paper. To the filtered broth 5 gm. of sodium chloride and 10 gm. of neopeptone per liter are added and the whole is brought to a temperature of 95°C. The pH is adjusted to 7.8 by adding twice normal NaOH. The broth is autoclaved at 15 pounds pressure for 10 minutes and is immediately filtered through grey French filter paper to remove the precipitate. 20 gm. of bacto-agar are added to each liter of broth and the mixture is autoclaved for 15 minutes under 15 pounds pressure. The agar is then filtered to remove the resulting precipitate and is poured in 12 cc. amounts into sterile tubes and these in turn are sterilized for 10 minutes under 15 pounds pressure. The final pH of the agar prepared in this fashion ranges from 7.4 to 7.6. We think that it is essential to use neopeptone in the preparation of the medium because in our experience ordinary peptones seem to inhibit the growth of the minute hemolytic streptococci.

The inoculated plates are incubated at 37°C. for 24 hours before being examined for the minute hemolytic areas which denote the presence of the organisms. In order to distinguish these areas, a good source of light is required, and on dark days we have found that the light from a substage microscope lamp serves very well as a source of illumination. If no areas of hemolysis are visible the plates are reincubated, with daily examinations, for an additional period of 72 hours before finally being discarded. If, however, suspicious areas of hemolysis are seen, they are examined for the presence of a colony under the low power of an ordinary microscope. Final identification of the colony is accomplished by fishing and transferring it to sugar-free beef infusion rabbit's blood broth in which, after 24 to 48 hours incubation at 37°C., growth turbidity and hemolysis will be evident if the organism is a minute hemolytic streptococcus. In fishing we have found that it is necessary to use a colonial microscope as otherwise mixed cultures will result.

Colonial Morphology, Hemolysis and Growth in Blood Agar.—In studying these characteristics the organisms were grown in sugar-free, 10 per cent, rabbit's blood agar poured plates at a temperature of 37°C. After 18, 24 and 48 hours, the size and morphology of the colony and the approximate diameter of the area of hemolysis were determined by studying colonies under the lower power of an

ordinary microscope. A standardized ocular micrometer was used in making the measurements.

Fermentation Reactions.—10 per cent sterile lactose, mannitol, salicin, trehalose and sorbitol solutions were added to tubes of sugar-free beef infusion broth in amounts sufficient to make the final concentration of these substances in the broth 0.5 per cent. The five sugar media were each inoculated with 0.1 cc. of an actively growing strain and were incubated for 4 days at 37°C. If an abundant growth occurred the presence or absence of the production of acid was determined by adding Andrade's indicator.

Reduction of Methylene Blue Milk.—R. C. Avery's (2) method of preparing the methylene blue milk medium was used. This medium with plain milk controls was inoculated by adding 0.1 cc. of an actively growing culture to 5 cc. of each medium. The cultures were incubated at 37°C. for 1 week and were examined each day for evidence of the reduction of methylene blue.

Hydrolysis of Sodium Hippurate.—The method described by Ayers and Rupp (3) was employed. The cultures were incubated at 37°C. for 4 days. Known positive cultures and uninoculated control tubes were included in each series.

Fibrinolytic Activity.—Dr. W. S. Tillett has tested a number of our strains for their fibrinolytic activity, using the method recently described by Tillett and Garner (4).

Final pH.—The final pH attained by the various strains of minute hemolytic streptococci after 3 days of growth in 1 per cent dextrose broth was determined by the methods of O. T. Avery and Cullen (5). Readings were made upon the supernatant broth of centrifuged cultures. Brom-cresyl green was the indicator used and the readings were checked against standards of brom-cresyl green phthalate-NaOH buffer solutions. All of our strains have been checked two or more times and where a difference of more than 0.2 pH was obtained the results were averaged. Because of the natural error in these determinations the results are expressed in terms of ± 0.1 pH.

RESULTS

Since the time of the initial isolation of the organisms from the throat of an individual ill with latent glomerular nephritis we have isolated and observed over 200 strains of minute *beta* hemolytic streptococci. These have been obtained from individuals ill with glomerular nephritis, rheumatic infection, other acute or chronic diseases and from normal human beings. Upon two occasions they have been recovered in pure culture from pus removed from acutely inflamed paranasal sinuses and in one instance from the pus from an abscess of the deep tissues of the chest.

In the primary isolation in rabbit's blood agar poured plates the

small areas of hemolysis are not usually evident until after 24 hours of incubation at 37°C. It is important that the blood agar plates be free from sediment and from bits of unmelted agar because these may easily be confused with true hemolysis. When the areas of hemolysis first become visible the colony cannot be distinguished by means of the unaided eye, and resort must be had to the use of the low power of an ordinary microscope. At this stage of development, the colony appears as a small, finely granular, roughly circular object ranging in size from 18 to 30 microns and surrounded by a relatively large area of true *beta* type hemolysis. Occasionally the colonies appear to be wrinkled and crenated or they may have a curious tetradic appearance. Rarely have the colonies been oval in the primary culture. By the end of 48 hours incubation they are visible to the naked eye, although, in certain instances, 96 hours of incubation were required before they were visible. In the first stages of development the ratio of the diameter of the area of the hemolysis to the diameter of the colony is roughly from 4 to 1, to 10 to 1. With further incubation this ratio decreases so that by the end of 48 hours the ratio is generally 3 or 4 to 1.

The growth from the first transfer of a colony to sugar-free rabbit's blood broth may be very slow. It is therefore advisable to incubate these cultures for at least 48 hours if no growth is visible at the end of 24 hours. The second transfer should also be made in a blood broth medium in order that the strain may become well established. In films made from cultures in liquid medium and stained by Gram's method the organisms appear as minute cocci occurring singly, in pairs, in short chains and in small and large masses. The individual coccus is one-half to two-thirds the size of the ordinary *beta* hemolytic streptococcus and it stains indifferently with Gram, some strains being strongly Gram-positive while others are Gram-negative.

The organisms grow sparsely in plain sugar-free beef infusion broth and are maintained with difficulty in such a medium. In 0.1 per cent dextrose beef infusion broth growth is generally diffuse and abundant in the first two or three subplants from blood broth but unless care is exercised the organisms soon tend to die. Sugar-free blood broth appears to be a good medium for carrying strains of them.

If one inoculates a loopful of these organisms upon the surface of

a sugar-free, 5 per cent, rabbit's blood agar plate and spreads the inoculum with a glass rod, a fine mist of colonies will be observed after 18 to 24 hours of incubation. Hemolysis may or may not be visible at this time. However, at the end of 48 hours incubation marked hemolysis is present. When well spaced the colonies have the following characteristics. They may be dewdrop, flat peaked or peaked in outline. They are generally smooth and shiny although strains showing glazed, granular or wrinkled surfaces have been encountered. The hemolyzed agar about the colony is frequently depressed and pitted and the colonies themselves are dry and tenacious, and are removed with difficulty from the surface. An incubation period of from 5 to 6 days is required before the colonies reach their maximum size.

These streptococci are facultative anaerobes and can be grown on blood agar in anaerobic jars or in Noguchi's medium. They are difficult to maintain in stock cultures because they die out rather rapidly in ordinary media when left either at room temperature or at a temperature of ±4°C. An easy method of preserving them is to inoculate Noguchi tubes containing 10 cc. of rabbit's blood broth with 0.1 cc. of culture and, after 24 hours of incubation at 37°C., to seal the tubes with sterile vaseline and to store them at ±4°C. Cultures may be maintained for at least 4 months under these conditions.

In passing we must state that in the course of their primary isolation in rabbit's blood agar plates we have noted other microorganisms which tend to produce very small colonies and small zones of hemolysis. Among these are small forms of *Hemophilus hemolyticus*, members of the *Neisseria* group, minute *alpha* hemolytic streptococci and a hemolytic Gram-positive coccus which is as yet unidentified. All of these forms may be confused with minute streptococci as far as their gross appearance in blood agar plates is concerned.

The minute streptococci we have studied tend to fall into two main groups, the first consisting of strains which ferment salicin and trehalose, and the second those which possess the *Streptococcus pyogenes* type of fermentation. Several strains showing irregular powers of fermentation were placed in a third group. We feel, however, that these strains represent examples of other groups which as yet have been encountered but rarely and we do not consider that these organ-

TABLE I

Cultural and Biochemical Characteristics of Group I Minute Hemolytic Streptococci

Strain	Source	Growth in blood agar			Fermentation of					Reduction of methylene blue	Hydrolysis of sodium hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
		Colony / Hemolysis in mm.										
		18 hr.	24 hr.	48 hr.	Lactose	Mannitol	Salicin	Trehalose	Sorbitol			
Hy. I	Normal	0.108 / 0.648	0.198 / 0.990	0.378 / 1.22	−	−	+	+	−	−	−	4.9
Hy. II	"	0.108 / 0.630	0.216 / 1.01	0.360 / 1.26	−	−	+	+	−	−	−	4.9
Ve.	"	−	−	0.162 / 0.752	−	−	+	+	−	−	−	4.8
S	"	−	−	0.252 / 0.774	−	−	+	+	−	−	−	4.8
My.	"	0.090 / 0.324	0.126 / 0.404	0.396 / 1.03	−	−	+	+	−	−	−	4.9
Wi.	"	−	−	0.198 / 0.540	−	−	+	+	−	−	−	4.8
Pa.	"	−	−	0.180 / 0.738	−	−	+	+	−	−	−	4.8
De.	"	−	−	0.126 / 0.900	−	−	+	+	−	−	−	5.0
St.	"	0.090 / 0.324	0.126 / 0.522	0.306 / 1.08	−	−	+	+	−	−	−	5.0
N	Nephritis	−	−	0.126 / 0.450	−	−	+	+	−	−	−	4.7
M.H.W.	"	0.045 / 0.234	0.045 / 0.252	0.144 / 0.576	−	−	+	+	−	−	−	4.8
Ha.	"	0.090 / 0.450	0.180 / 0.900	0.396 / 1.29	−	−	+	+	−	−	−	4.9

TABLE I—*Continued*

Strain	Source	Growth in blood agar: Colony / Hemolysis in mm. 18 hr.	24 hr.	48 hr.	Fermentation of: Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Reduction of methylene blue	Hydrolysis of sodium hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
103 H	Nephritis	–	0.054 0.288	0.244 0.828	–	–	+	+	–	–	–	4.8
Me.	"	–	–	0.244 0.576	–	–	+	+	–	–	–	4.9
Ho.	"	–	0.054 0.324	0.274 0.864	–	–	+	+	–	–	–	4.8
65 F	"	–	–	0.396 0.990	–	–	+	+	–	–	–	4.9
Kl. I	"	0.072 0.378	0.108 0.540	0.292 1.15	–	–	+	+	–	–	–	4.8
Kl. II	"	0.054 0.360	0.090 0.540	0.256 1.08	–	–	+	+	–	–	–	4.8
Me.	"	–	0.108 0.425	0.288 0.936	–	–	+	+	–	–	–	5.0
W	"	–	–	0.180 0.720	–	–	+	+	–	–	–	4.7
Pai.	"	–	–	0.198 1.08	–	–	+	+	–	–	–	4.7
D	"	–	0.072 0.360	0.306 1.22	–	–	+	+	–	–	–	4.7
40 E	"	0.027 0.234	0.054 0.324	0.126 0.468	–	–	+	+	–	–	–	4.9
Wh.	Rheumatic fever	–	0.126 0.378	0.162 0.844	–	–	+	+	–	–	–	4.7
Ta.	" "	–	–	0.126 0.504	–	–	+	+	–	–	–	4.7

TABLE I—*Concluded*

Strain	Source	Growth in blood agar: Colony / Hemolysis in mm.			Fermentation of					Reduction of methylene blue	Hydrolysis of sodium hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
		18 hr.	24 hr.	48 hr.	Lactose	Mannitol	Salicin	Trehalose	Sorbitol			
An.	Acute tonsillitis	0.090 / 0.414	0.144 / 0.720	0.270 / 0.882	−	−	+	+	−	−	−	4.8
Ba.	" "	−	0.054 / 0.360	0.270 / 1.01	−	−	+	+	−	−	−	4.8
Ch.	" "	0.108 / 0.558	0.162 / 0.684	0.270 / 0.900	−	−	+	+	−	−	−	4.9
Jo.	Scarlet fever	0.054 / 0.360	0.090 / 0.521	0.252 / 1.04	−	−	+	+	−	−	−	4.8
Fo. I	Sinusitis (antrum)	−	0.126 / 0.404	0.198 / 0.720	−	−	+	+	−	−	−	4.6
Fo. II	Sinusitis (ethmoid)	−	0.090 / 0.450	0.216 / 0.756	−	−	+	+	−	−	−	4.6
38 E	Phlebitis	0.054 / 0.216	0.090 / 0.414	0.180 / 0.918	−	−	+	+	−	−	−	4.9
Wr.	Abscess of chest wall	0.027 / 0.180	0.036 / 0.288	0.270 / 0.864	−	−	+	+	−	−	−	4.9
95 E	Diabetes	−	0.036 / 0.252	0.144 / 0.604	−	−	+	+	−	−	−	4.8

isms constitute a true group in the same sense as do the organisms in Groups I and II.

The majority of the strains fall into the first group. It is apparent from Table I that these strains tend to produce rather small colonies and in some instances such a retarded type of hemolysis that neither colony nor hemolysis are visible at the end of 24 hours. At the end of 18 hours incubation, with certain strains, the ratio of the diameter of the zone of hemolysis to the diameter of the colony varied from

4 to 1, to 9 to 1. At the end of 48 hours of incubation this ratio was in most instances 3 or 4 to 1. Ordinary *beta* hemolytic streptococci from human sources have a ratio of 3 or 4 to 1 from the time the colonies are first visible and preserve this ratio throughout the period of incubation.

All of the strains of minute hemolytic streptococci in this group fermented salicin and trehalose while none fermented lactose, mannitol or sorbitol. On the basis of their reaction in lactose, mannitol and salicin, they would be classed as belonging to the *Streptococcus equi* group. However, Edwards (6) has recently shown that almost all hemolytic streptococci of human origin ferment trehalose and not sorbitol while the majority of hemolytic streptococci from animal sources ferment sorbitol and not trehalose. An exception to this latter finding exists in the hemolytic streptococci which are isolated from cases of strangles in horses, which ferment neither trehalose nor sorbitol but do attack salicin. Therefore, while the organisms in this first group resemble typical equine streptococci in their ability to ferment salicin, their power of fermenting trehalose definitely indicates that they cannot be classed as typical *Streptococcus equi* strains.

Lancefield (7) has demonstrated by means of a precipitin reaction that the strains of *beta* hemolytic streptococci which ferment trehalose can be divided into two groups when tested by this serological method. In her Group A almost all of the strains are of human origin while in Group B many of the strains are derived from bovine sources. However, the Group B strains hydrolyze sodium hippurate and produce more acid in 1 per cent dextrose broth than do the Group A strains. None of the minute organisms hydrolyzed sodium hippurate but they differed from Lancefield's Group A strains in acid production in 1 per cent dextrose broth. Many of our organisms produced more acid than the Group A strains but less acid than the Group B strains.

Strains of minute hemolytic streptococci possessing a *Streptococcus pyogenes* type of fermentation were placed in a second group. A marked variation in the ratio of the diameter of the zone of hemolysis to the diameter of the colony was noted among the members of this group. Certain strains behaved in this respect as did members of the first group while others maintained a high ratio throughout the period of incubation. All fermented lactose, salicin and trehalose.

None hydrolyzed sodium hippurate or reduced methylene blue milk. A moderate amount of acid was produced in 1 per cent dextrose broth by the members of this group.

TABLE II

Cultural and Biochemical Characteristics of Groups II and III Minute Hemolytic Streptococci

Strain	Source	Growth in blood agar: Colony/Hemolysis in mm. 18 hr.	24 hr.	48 hr.	Fermentation of: Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Reduction of methylene blue	Hydrolysis of sodium hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
					Group II							
Te. I	Normal	0.108/0.954	0.162/1.48	0.234/2.52	+	−	+	+	−	−	−	4.6
Te. II	"	0.108/0.990	0.162/1.67	0.234/2.34	+	−	+	+	−	−	−	4.5
Te. III	"	0.108/0.540	0.180/0.900	0.396/2.34	+	−	+	+	−	−	−	4.7
Ha. I	"	0.108/1.01	0.180/1.30	0.270/2.25	+	−	+	+	−	−	−	4.8
Ha. II	"	0.108/1.01	0.162/1.28	0.198/2.25	+	−	+	+	−	−	−	4.8
Ma.	"	0.126/1.01	0.180/1.98	0.352/2.97	+	−	+	+	−	−	−	4.8
Ru.	"	0.090/0.270	0.126/0.720	0.270/0.846	+	−	+	+	−	−	−	4.9
Pe.	Scarlet fever	0.072/0.540	0.108/1.55	0.180/3.24	+	−	+	+	−	−	−	4.7
Ha.	Broncho-pneumonia	0.090/0.342	0.144/0.504	0.342/0.954	+	−	+	+	−	−	−	4.9
86 E	Tuberculous kidney	0.072/0.432	0.180/0.900	0.396/1.35	+	−	+	+	−	−	−	4.8

TABLE II—*Concluded*

Strain	Source	Growth in blood agar: Colony / Hemolysis in mm. 18 hr.	24 hr.	48 hr.	Fermentation of: Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Reduction of methylene blue	Hydrolysis of sodium hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
					Group III							
61 E	Nephritis	—	0.900 / 0.360	0.180 / 0.720	—	—	—	—	—	—	—	5.4
106 E	Nephritis	—	—	0.180 / 0.540	—	—	+	—	—	—	—	5.2
101 F	Rheumatic fever	—	—	0.234 / 0.684	—	—	—	—	—	—	—	5.4
45 E	" "	0.072 / 0.432	0.126 / 0.612	0.360 / 1.02	—	—	—	+	—	—	—	5.2
22 E	Acute pharyngitis	—	0.126 / 0.360	0.270 / 0.900	—	—	+	—	—	—	—	4.9
84 E	" "	—	—	0.378 / 1.11	—	—	+	—	—	—	—	5.2

The third group comprised strains which possess weak powers of fermenting the test sugars. Three of the members of this group had the typical *Streptococcus equi* type of fermentation reactions, and the other three resembled *Streptococcus subacidus* as far as their fermentation reactions in lactose, mannitol and salicin were concerned. None of the six strains hydrolyzed sodium hippurate or reduced methylene blue milk and with one exception only a small amount of acid was produced in 1 per cent dextrose broth by the members of this group.

A certain number of strains from the first two groups have been tested for the presence of the fibrinolytic substance recently described by Tillett and Garner (4) but as yet none of the tested strains has shown this substance. Inasmuch as Tillett (8) has demonstrated that 98.8 per cent of *beta* hemolytic streptococci from human sources possessed this substance while only 11 per cent of *beta* hemolytic strepto-

cocci of animal origin were capable of bringing about fibrinolysis, it is apparent that the lack of the fibrinolytic substance in the minute streptococci further differentiates them from ordinary hemolytic streptococci of human origin. Preliminary studies of the antigenic structure of these organisms have shown that, upon the basis of Lancefield's precipitin reaction, they fall into two groups, which are different from those previously described (7). This observation further separates the minute streptococci from ordinary *beta* hemolytic streptococci.

DISCUSSION

It is evident from this study of the cultural and biochemical properties of these minute hemolytic organisms, that, while they resemble ordinary *beta* hemolytic streptococci in many respects, they cannot be classified under any previously described groups of these latter organisms.

The individual organisms are smaller than ordinary hemolytic streptococci, and the colonies are much more minute. Their fermentation reactions in the test sugars resemble those described for ordinary hemolytic streptococci of human origin except in three instances in which they conformed with those described for the organisms isolated from strangles in horses. None of the strains hydrolyzed sodium hippurate nor did they reduce methylene blue milk. The majority of the strains produced a fair amount of acid when grown in 1 per cent dextrose broth. None of the tested strains showed the presence of the fibrinolytic substance. Thus, while all of the strains possess certain of the characteristics of ordinary *beta* hemolytic streptococci none of them completely conform with known species of these organisms.

We realize that the term "minute" hemolytic streptococci is probably not the best one that could be applied to the organisms which we have studied but from a descriptive and differential point of view this term serves the purpose of pointing out a striking characteristic of this group. It is likely that they represent either new species of the *beta* hemolytic streptococcus or that they are hitherto undescribed variants of the ordinary *beta* hemolytic streptococci. At present we are unable to give a final opinion upon this point and can only state

that we have not observed a reversion of these minute organisms to the larger forms of *beta* hemolytic streptococci.

CONCLUSIONS

The cultural and biochemical characteristics of a group of minute hitherto undescribed *beta* hemolytic streptococci from human sources have been recorded and upon the basis of these cultural reactions it is suggested that these organisms may represent new species of the genus streptococcus.

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