SECTION IV

STREPTOCOCCI AND PNEUMOCOCCI

Cultures of Gram positive cocci in pairs and chains received by the laboratory are inoculated into Trypticase Soy (Media Manual #1726) or Todd Hewitt Broth (Media Manual #1711) and Thioglycollate Broth (Media Manual #1708), and streaked on two blood agar plates (Media Manual #1310, plus 5 percent defibrinated Rabbit Blood), incubated at 35°, aerobically and in a candle jar. Many Streptococci, and the pneumococcus, are by preference somewhat microaerophilic, and more vigorous growth is usually obtained in the candle jar.

After overnight incubation, the broths and plate growth are smeared and Gram stained, and the plates are inspected microscopically for hemolysis. If the cocci show beta or gamma (no) hemolysis, proceed at once to streptococcal identification as described in Section A below. However, if the CO_2 blood plate shows alpha (viridans) hemolysis, the pneumococcus must also be considered; refer to Section B.

I. STREPTOCOCCI

A. Hemolysis

The primary differentiation among the streptococci is based on establishing the type of hemolysis, and this is one of the earliest steps in streptoccal classification. Hemolysis is best studied in a pour plate, with subsequent microscopic examination of the subsurface colonies.

To prepare a pour plate for the study of hemolysis, proceed as follows:

1. Melt a tube of Neopeptone Infusion Agar (Media Manual #1517) in the Arnold; hold in 52° water bath.

2. Pipette 15 ml sterile 0.85 percent NaCl into a sterile 20 x 125 screw cap tube.

3. Transfer 1 drained loopful of a well-grown 18-24 hour culture of the unknown Streptococcus in Todd Hewitt or Trypticase Soy Broth to the tube of sterile saline. (Step 2) Mix well. the culture dilution should be prepared not more than 15 minutes before use in the pour plate.

4. Add 0.6 - 0.8 ml sterile defibrinated rabbit blood to the tube of melted agar (Step) and mix gently, avoiding bubbles.

5. Add 1 drained loopful of the culture dilution (Step) to the tube of melted blood agar, mix gently but thoroughly, and pour into a petri plate. Allow to harden, then incubate at 35°, usually for 24 hours. Although some Streptococci require 48-72 hours to grow sufficiently to demonstrate their proper hemolytic capacities.

6. Examine the incubated pour plate under low power of the microscope (100x magnification). Interpretation of the observations and a discussion of the various types of hemolysis, varieties of hemolysins produced by beta streptococci and problems involved in the study of hemolysis may be found in the CDC communication "General Considerations: Hemolysis," which follows.

GENERAL CONSIDERATIONS: HEMOLYSIS (From CDC)

The isolation and identification of the Gram-positive cocci is contingent initially upon the ability of the laboratorian to differentiate alpha, beta, and gamma hemolysis on blood agar media. To demonstrate hemolysis, laboratorians often used the streaked blood plate with subsequent incubation under aerobic conditions. Unfortunately, the convenience offered by this technique is more often than not offset by the difficulty introduced in differentiating hemolytic activity. Hemolysis is a more complex system than generally recognized and is influenced by a number of factors--some inherent and some imposed inadvertently by the laboratorian. The differentiating of hemolysis, then, is dependent upon an understanding of the phenomenon. Some topics which require particular examination and understanding will be presented in this text. In addition, some specific suggestions will be offered to aid in the determination of hemolytic activity.

A. Definitions of Kinds of Hemolysis
B. Properties of Streptolysin O and S
C. Influence of Various Bloods on Hemolytic Expression
D. Influence of the Basal Medium
E. Specific Recommendations

1. Definitions of Kinds of Hemolysis

Reference to hemolysis in this text will be based on the classification of Brown as postulated in the monograph of 1919 and apply specifically to the characterization of streptococci. The types of hemolysis are defined as follows:

Alpha (a) - An indistinct zone of partial destruction of red blood cells about the colony, often accompanied by a greenish to brownish discoloration of the medium.

Beta (B) - Clear, colorless zone around the streptococcus colonies in which the red blood cells have undergone complete dissolution. Particular attention should be directed to the spindle shaped subsurface colonies surrounded by a definite zone of beta hemolysis. The medium surrounding the subsurface colony is entirely free of intact red blood cells.

Gamma (y) - No apparent hemolytic activity or discoloration produced about the colony. Gamma hemolysis is characterized by the absence of disrupted or discolored red blood cells.

Alpha prime (') or Wide Zone Alpha (WZa) - A small halo or envelope of intact or partially lysed red blood cells lying adjacent to the bacterial colony, with a zone of complete hemolysis extending further out into the medium. When examined macroscopically, alpha hemolysis can be confused with beta hemolysis.

These observations by Brown were based on the microscopic examination of subsurface colonies in blood agar pour plates. Through the years, these definitions have been applied to the characterization of colonies arising on the surface of streaked blood plates. This extended application has not been achieved smoothly because of the character of the hemolysins responsible for beta hemolysis and the misinterpretation of alpha prime as beta hemolysis.

2. Properties of Streptolysin O and S on Blood Plates

In 1932, and subsequently, Todd described two distinct hemolysins responsible for beta hemolytic activity. He differentiated these hemolysins on the basis of antigenicity and susceptibility to inactivation by oxidation. He designated the antigenic, oxygen labile hemolysin as streptolysin O and the nonantigenic, oxygen-stable hemolysin as streptolysin S. It was noted that oxygen sensitive streptolysin O could be reactivated in the presence of reducing agents. Further, streptolysin S was found not to be produced

in serum free broth, and its production was inhibited in media rich in fermentable carbohydrate. Keeping these restrictive properties in mind, it becomes obvious that aerobic incubation of streaked blood plates neutralizes the hemolytic activity of streptolysin O and restricts the characterization of the beta hemolytic streptococcus to streptolysin S activity which may vary greatly from strain to strain. By using streaked blood plates incubated in the presence of atmospheric oxygen, the investigator imposes limitations upon the hemolytic expression of the organism which could cause the beta hemolytic streptococcus to be overlooked.

3. Influence of Various Bloods on Hemolysis

Much has been intimated in the literature about the influences of blood obtained from a variety of animal species on the hemolytic expression of streptococci. The inference that the type of hemolysis changes dramatically from alpha to beta or beta to gamma, depending on the species of blood incorporated into the medium, is frightening in its implications. Although there is some basis for these disquieting revelations, it should be emphasized that presently available information indicates that variations in hemolytic activity are restricted to the enterococci. Updyke (1957) reported the following:

"In a study at the CDC 100 Group A strains and 45-50 each of Groups B, C, and G strains grew equally well and gave identical hemolytic reactions in pour plates prepared from sheep, rabbit, horse, and human blood bank blood. The only differences in hemolytic activity occurred among the enterococci: 88 percent of 90 Group D strains were alpha hemolytic in sheep blood agar and beta hemolytic in rabbit, horse, and human blood agar ..."

Thus, the changes in beta hemolysis in blood from different animals is usually manifested (except for the enterococci) as a variation in size of the hemolytic zone, sharpness of the edge of the zone, and slight opacity <u>not</u> due to incomplete hemolysis. The variations in alpha hemolysis are much more pronounced and, for this reason, misinterpretation of alpha hemolysis accounts for much of the confusion in the literature. Differences in alpha hemolysis in a variety of blood media may range from delicate variations in intensity of greening on one hand to the demonstration of alpha prime hemolysis on the other. Alpha prime hemolysis, on cursory examination, may be confused with beta hemolysis and can be differentiated with certainty only be microscopic examination of subsurface colonies in pour plates.

In recent years, investigators have recommended that sheep blood be incorporated in blood agar plates for the isolation of beta hemolytic streptococci. Sheep blood does not support the growth of most strains of Haemophilus; of particular importance, it does not support beta hemolytic Haemophilus haemolyticus, which tends to confuse the isolation of streptococci. Conversely, some investigators feel that failure of sheep blood to support growth of Haemophilus is a distinct disadvantage of sheep blood. They argue that inability to isolate Haemophilus on sheep blood necessitates the use of a second blood medium to augment the sheep blood plate. It must be recognized that use of rabbit or horse blood as an alternative to sheep blood requires that particular care be exercised in the selection of beta hemolytic colonies for characterization and that all colonies be Gram stained.

The use of outdated human blood from blood banks is widespread particularly in hospital and clinical laboratories. This practice is not encouraged because the donor may have had recent antibiotic therapy, and a residual level in the blood might have deleterious effects upon streptococcal isolates. Finally, the anticoagulant employed in the blood bank could have toxic side effects on bacterial isolates. Streptococci are inhibited by anticoagulants such as oxalate and citrate. If human blood must be employed in plating media, it is imperative that each new blood medium be tested for the ability to recover streptococci and, further, that these streptococci demonstrate characteristic hemolytic traits.

4. Influence of Basal Medium

In selecting a medium for use as a blood agar base, choose an infusion medium that is free of fermentable carbohydrate and has a final pH of 7.3 - 7.4 before addition of the blood. The presence of dextrose in the basal medium for blood plates results in the inhibition of hemolysis by beta hemolytic streptococci. The deleterious effect of dextrose upon hemolytic activity may be partially explained by inactivation of streptolysin S by the acid produced during fermentation. Brown stated that whereas small amounts of dextrose were beneficial to demonstration of alpha hemolysis, actively fermentating gamma hemolytic stains produced discoloration of the blood medium which confused the differentiation of the types of hemolysis.

5. Specific Recommendations

Plate the specimen upon media and under growth conditions which favor the demonstration of streptococcal characteristics. Inoculate the specimen into a carbohydrate free infusion medium, pH 7.3 - 7.4, containing 4-5 percent defibrinated sheep blood, preferably. Ideally, the specimen should be plated in a our plate to allow for microscopic examination of subsurface colonies. The suspect colony should be examined under low power magnification (total magnification 100X) using a conventional microscope. It is imperative that the edge of the colony be in sharp focus during the determination of hemolytic activity. A portion of the surface of the hardened pour plate can be streaked in the usual manner to permit ease of isolation of the obvious beta colonies. If streak plates remain the method of choice, at the very least, stab the streaked plate with the inoculating loop to get subsurface growth and to assure streptolysin O and S participant. The plates should be incubated under conditions of reduced oxygen tension such as in a candle jar which attains 3-5 percent CO_2.

II. PRESUMPTIVE IDENTIFICATION OF STREPTOCOCCI (From CDC 8-30-74)

A. Introduction

The best method of identifying the streptococci is by growing isolated pure colonies of the infecting organism, extracting the group carbohydrate, and demonstrating a serological reaction between the extracted antigen and specific grouping antiserum. The CDC recommends the serological groping procedure as the method of choice for identifying the streptococci. However, we realize that this method of growing and extracting the organism is time consuming, and the cost of obtaining specific potent antisera makes the method unacceptable for some laboratories. An alternative method of identifying the pathogenic streptococci should be considered by the laboratories that are not performing serological grouping. This alternative method is based on determination of 1) hemolytic activity, 2) bacitracin susceptibility, 3) hydrolysis of esculin in the presence of 40 percent bile, 4) tolerance to 6.5 percent NaCl broth, and 5) hydrolysis of sodium hippurate. These tests, when run properly with adequate controls, will accurately identify more than 95 percent of the pathogenic streptococci from clinical material. The resulting identifications are presumptive, and reports should indicate this fact. The formulas for the media used in these determinations, test procedures, and analyses of results will be discussed below.

B. Hemolysis

Although determination of hemolysis is the most important procedure in identifying pathogenic streptococci, we will not describe the methods for determination. Many publications and manuals have described this hemolysis (1, 2) and have given the pros and cons of various bases and bloods that can be used in agar plates. We advise using the pour-streak plate method with sheep blood in any infusion agar base that is free of fermentable carbohydrate and with a final pH of 7.3 to 7.4. Subsurface colonies should be examined with a microscope to determine hemolysis. Rabbit or horse blood may be substituted in the base, but the user should be aware of different reactions that can result (2, 3). A description is given of how the surface of a blood agar plate is streaked and stabbed in an alternative method suggested by the American Health Association (4). The stabbed area is observed with a microscope as it is in the pour-streak method. Although this method is not as acceptable as the pour-streak method, it can be used when making pour-streaked plates is impossible.

References:

1. Brown, J. H. Rockefeller, University Monograph No. 9, 1919

2. Taranta, A. and M. D. Moody, Ped. Clin. N. A. 18:125-143

3. Updyke, E. L., Pub. Hlth. Lab. 15:78 (1957)

4. Wannamaker, L. W., 1965. a method of culturing beta-hemolytic streptococci from the throat. AHA Statement EM 164.

C. Bacitracin ([redacted])

Commercial discs are available that will differentiate between beta-hemolytic group A streptococci and other beta-hemolytic streptococci. the users of bacitracin discs should be aware of several important factors. 1) Be sure to purchase differential, not sensitivity discs. Discs sold and used for bacitracin susceptibility testing have too high a concentration of bacitracin to accurately differentiate between group A and nongroup A streptococci. 2) A heavy inoculum of a pure culture is advisable. One report of placing the differential disc on primary plates that had been inoculated with throat swabs resulted in only a 70 percent accuracy of identification for group A streptococci (6). The test has been designed for use with pure cultures, not mixed cultures (3, 4). 3) The test is designed for differentiating beta-hemolytic streptococci. Correct determination of hemolysis must be achieved before this differential test can be reliable. Many alpha-hemolytic