5) Place in oil bath at 150° C for 15 minutes.
6) Cool. Add 0.25 ml of acid-alcohol. Acid-alcohol is made with 95 parts anhydrous (100%) alcohol and 5 parts 2N HCl. Shake tube to mix.
7) Centrifuge and decant supernatant to small tube; keep supernatant.
8) Add 0.25 ml acetone; shake tube to mix.
9) Centrifuge and discard supernatant fluid; keep the precipitate.
10) Add 1 ml of saline and one drop of phenol red indicator to the precipitate. Shake and neutralize with a trace of sodium carbonate powder. If after shaking a part does not dissolve, centrifuge and neutralize supernatant.
11) React with grouping antisera.

(c) Rantz and Randall's autoclave extraction.

1) Cells for extraction are grown in 30 ml of Todd-Hewitt or suitable broth. The broth is inoculated and incubated overnight (16-24 hours) at 35-37° C.
2) Pack the cells by centrifugation.
3) Discard the supernatant fluid; save the cells.
4) Add 0.5 ml of 0.85% NaCl solution. Shake to suspend the cells.
5) Autoclave the tube and cells for 15 minutes at 121° C.
6) Centrifuge to sediment the cellular debris.
7) Decant the supernatant fluid into clean sterile container; discard sediment.
8) React with grouping antisera.

(d) El Kholy's nitrous acid extraction.

1) Cells for extraction are grown on a blood agar plate or in a 5-ml Todd-Hewitt or other suitable broth. The medium is inoculated and incubated overnight (16-24 hours) at 35-37° C.
2) If a 5-ml broth is used, recover the cells by centrifugation, and add one drop of saline to the packed cells. If an agar plate is used, recover the cells by adding one or two drops of saline to the plate, scraping the growth free, and transferring suspension to a small tube.
3) Add two drops of 4 molar $NaNO_2$ solution to the cell suspension; 4 M $NaNO_2$ is made by adding 276g $NaNO_2$ per litre of distilled water.
4) Add 1 drop of acetic acid, mix well.
5) Allow to react for 15 minutes at room temperature.
6) Add one drop of metacresol purple indicator and adjust to pH 7.4 with N NaOH.
7) Centrifuge to clarify.
8) React with grouping antisera.

(e) Maxted's Streptonyces albus enzyme extraction method.

1) Strains are grown on a blood agar plate, 16-24 hours at 37° C.
2) Pipette 0.25 ml of enzyme solution (Streptomyces albus enzyme; available commercially) into a small test tube, 12 x 75 mm or smaller.
3) Scrape a large loopful of growth from the blood agar plate and suspend in the enzyme solution.
4) Place in a 45° C water bath until solution is clear (about 90 minutes).
5) Cool to room temperature and centrifuge for 10 minutes at 2,000 r/min.
6) Decant into a clean container.
7) React with grouping antisera.

S. albus enzyme should be resuspended in the volume according to the manufacturer's suggestions. Aliquetes should be stored at -20 to -70° C until used.

(f) Ederer's pronase B enzyme extraction.

1) Strains for extraction are grown on a blood agar plate. The plate is inoculated and incubated overnight (16-24 hours) at 35-37° C.
2) Prepare borate buffer with 0.01 Ca Cl_2.
   (a) Borate solution: 12.404 g boric acid (H_3BO_3) is dissolved in 100 ml of N NaOH and diluted to 1 litre with distilled water.
   (b) 0.1 N HCl.
   (c) 1 molar $CaCl_2$.

Add 525 ml of borate solution to 475 ml of 0.1 N HCl and 10 ml of M $CaCl_2$.

3) Prepare pronase solution. 20 mg per ml of pronase B is prepared in borate buffer. The solution is dispensed in 0.5-ml portions in 13- x 75-mm tubes; the tubes are corked, and the solution is frozen at -20° C until used.

4) Remove all the growth from the plate by using a swab. Place swab in buffered enzyme solution and rotate and squeeze as dry as possible by placing the swab against the side of the tube. The suspension should be cloudy.

5) Place at 35 to 45° C for 2 hours.

6) Centrifuge for 15 to 30 minutes in a clinical centrifuge.

7) Decant supernatant into clean sterile container.

8) React with grouping antiera.

Note: Aliquots of the enzyme solution should be stored at -20 or -70° C until used.

(g) Watson's S. albus-lysozyme enzyme extraction.

1) Strains for extraction are grown on a blood agar plate. The agar is inoculated and incubated overnight (16-24 hours) at 35-37° C.

2) Prepare enzyme mixture as follows: Place 5 mg per ml of lysozyme in distilled water. A convenient volume is 25 mg in 5 ml of distilled water, since S. albus enzyme is prepared to be resuspended in 5 ml of distilled water. Use the 5 ml of lysozyme solution to resuspend the S. albus enzyme. Centrifuge the solution to clarify and store in 0.5 ml quantities in 10- x 75-mm cork-stoppered tubes at -20° C.

3) Remove the growth from the blood agar plate with a sterile swab and transfer to the enzyme solution (0.5 ml). Mix the swab with the solution and squeeze it against the side of the tube to remove as much of the moisture as possible. Discard the swab.

4) Incubate the enzyme-cell mixture in a water bath at 40-45° C for 90 minutes.

5) Centrifuge to clarify and decant into a clean container.

6) React with grouping antisera.

C. THE PRECIPITIN TEST

1. The CDC test compared to Dr Lancefield's original technique

The CDC capillary ring precipitin test is shown in Figure 7. Note the cloudy reaction at the interface of the two reagents (group A tube). It is important to follow the directions of the test given below because of the nature of the antisera that are available.

The CDC technique differs from Dr Lancefield's technique as originally described in that the antigen (extract) is layered over the antiserum rather than vice versa. Dr Lancefield's technique works very well with antisera of high potency, but it does not work as well as the CDC technique with serum of low potency. Since the potency of most lots of CDC and commercial antisera is low, the CDC technique should be used.

2. Instructions for performing the CDC precipitin test for streptococcal grouping.

(a) Dip capillary tube (vaccine capillary tube with 1.2- to 1.5-mm outside diameter, Kimble borosilicate glass, both ends open, and lightly fire-polished) into serum (in screwcap vial) until a column about 1 cm long has been drawn in by capillary action. (To maintain sterility of the sera, sterilize the tubes and keep them sterile at the lower end until after the serum is taken up).

(b) Wipe off tube with facial tissue, taking care to hold tube so air does not enter the end.

(c) Dip tube into extract until an amount equal to the serum column is drawn up. If an air bubble separates serum and extract, discard tube and repeat.

(d) Wipe tube carefully. Fingerprints, serum, or extracts on the outside of the tube may simulate or obscure a positive reaction.

(e) Plunge the lower end of the tube into the plasticine until a small plug fills the opening. Do not let the reactants mix. The plasticine plug (at the same end of the tube as the reactants) will hold the reactants in place while the tube is inverted.

(f) Invert tube and insert gently into the plasticine-filled groove of the rack.

(g) After a few minutes (5 to 10), examine with a bright light against a dark background. A white cloud or ring at the center of the column represents a positive result. A strong reaction appears in 5 minutes; a weaker reaction develops more slowly. Since after 30 minutes the reaction may fade or a false positive may appear, examine the capillary tubes at frequent intervals between 10 and 30 minutes.

D. FLUORESCENT ANTIBODY (FA) GROUPING OF STREPTOCOCCI

1. Reliability of test for various streptococcal groups

The technique has been widely used to identify group A streptococci from throat swabs. Hundreds of thousands of cultures are examined each year for group A by this technique. The two chief advantages of the FA technique are that it is rapid and does not require pure cultures. The FA technique is the most convenient and reliable method of identifying group A streptococci when large volumes of throat swabs are processed.

Although FA reagents are available for group B, C, D, F, and G streptococci, these reagents are not recommended because of their low potency and cross-reactions. Even if the cross-reactions of the various reagents are known for certain lots, this knowledge is of limited value because the cross-reactions are not consistent between lots; therefore, each lot needs to be tested for cross-reactions. This is not practical. At present, the FA technique is recommended only for the identification of beta-hemolytic group A streptococci. There are several good sources of group FA reagents.

Figure 8 shows a group A streptococcal culture stained by group A FA antisera. The bright, light-coloured cells are apparent in short chains and single cells. The standard procedure for preparing the FA slides is described below. Techniques for direct examination of throat swabs without enrichment have not been developed. The swab must be placed in a suitable broth for a minimum of 2 hours before a culture can be stained.

2. Preparing and fixing the smears

(a) Place swabs in 1 ml of broth and incubate for 2 to 5 hours at 35 to 37° C.

(b) Remove the swab from broth and place in a sterile tube. If hemolysis is to be determined, use swab to prepare a streak-stab plate.

(c) Centrifuge broth for 5 minutes at approximately 2,000 r/min.

(d) Pour off broth into disinfectant solution and wipe lid with disinfectant-soaked cotton; resuspend cells in 1 ml of sterile buffered saline, pH 7.5 and recentrifuge.

(e) Pour off buffered saline into disinfectant solution, and while tube is in inverted position, remove all visible diluent by wiping lip with disinfectant-soaked cotton.

(f) Place tube in rack for 2 to 3 minutes and let residual buffered saline collect in bottom of tube. (Usually no additional diluent will be needed.)

(g) Mix cells in diluent.

(h) With a Pasteur capillary pipette, transfer sediment to area within circles on a FA slide. Try to transfer most of the sediment to the smears.

(i) Let smear dry in air. If atmosphere is humid, smears may be dried in an incubator at 35 to 37° C.

(j) Cover each smear with 95% ethanol. Keep wet for 1 minute; then let ethanol evaporate. After smears have thoroughly dried, they may be stained, or they may be frozen and stored and stained at a later date, if absolutely no thawing occurs in the interim.

3. Staining smears

(a) Cover smears nearest etched end of slide with small drop of control conjugate (normal rabbit globulin labeled with fluorescein-isothiocyanate and treated to remove nonspecific staining). Cover the other smear with group A antistreptococcus conjugate (Group A antistreptococcus globulin labeled with fluorescein-isothiocyanate and treated to remove nonspecific staining). Spread each conjugate over entire smear with an applicator stick held in a horizontal position. After the desirable sensitivity and specificity of a particular vial of group A antistreptococcus conjugate have been established with groups A, C, and G Streptococcus and Staphylococcus aureus control cultures, routine staining with the rabbit globulin control conjugate can be omitted.

(b) Let stand for 30 minutes at room temperature in a moist chamber. Half of a 15-cm Petri dish fitted with moist filter paper makes a suitable chamber.

(c) Shake excess conjugate onto disinfectant-soaked paper towel.

(d) Dip slides momentarily into buffered saline, pH 7.5, in a staining dish.

(e) Transfer to a second vessel of buffered saline and let stand for 10 minutes.

(f) Dip momentarily into distilled water and air-dry.

(g) Add a drop of buffered glycerol saline mounting fluid and a cover slip. Try to keep bubbles from forming in mounting fluid. Apply one drop of nail polish (or similar adhesive) to each corner of the cover slip.

4. Storing and examining the smears

Stained and mounted smears may be refrigerated (not frozen) and examined anytime within 24 to 48 hours without significant loss of brilliance of fluorescence. Smears to be used for reference purposes may be stored in a refrigerator for longer periods, although staining may become less intense. Slides to be stored in a refrigerator overnight or longer should be sealed completely with nail polish. The recommended filter system is a BG12 exciter and a OG1 barrier. The fluorescense should be estimated as follows:

4+ Maximal fluorescence, brilliant yellow-green clear-cut cell outline; sharply defined nonstaining centre of cell. Figure 8.

3+ Less brilliant yellow-green fluorescence; clear-cut cell outline; sharply defined nonstaining centre of cell.

2+ Less brilliant, but definite fluorescence; less clear-cut cell outline; nonstaining centre area fuzzy.

1+ Definite fluorescence, but very subdued; peripheral and centre staining at same intensity.

A satisfactory working dilution of the test reagent is one which stains all of the group A strains at 3+ or 4+ fluorescence intensity and the other strains at not more than 1+ or 2+. Occasionally, S. aureus, group C and group G streptococci, and, less frequently, group A streptococci stain at low levels.

5. Modifications of the FA technique

Modifications of the original FA procedure have been examined in three well-designed, recently reported studies. The modified procedures were compared with an acceptable standard technique; paired specimens and large numbers of cultures were processed. All the

modifications involve lessening the staining time of the FA reagent. These modifications ar summarized in Table 7. All of these modifications appear to be satisfactory and may be appropriate when the FA procedure is being used to identify group A streptococci.

E. GROUPING BY COUNTERIMMUNOELECTROPHORESIS (CIE) AND COAGGLUTINATION (CA)

These two recently developed techniques have been proposed for use as tools to aid in th serological identification of streptococci. The CIE and CA techniques are still in the experimental stage of development. At present, we cannot recommend either technique for routine use in the clinical laboratory. Interested microbiologists should consult the references in the next section of this manual, including our review in the CRC Press' Critical Reviews in Clinical Laboratory Science.

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PART III

PRESUMPTIVE IDENTIFICATION OF STREPTOCOCCI BY NONSEROLOGICAL METHODS

A. INTRODUCTION

The best method for 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. CDC recommends the serological grouping procedure as the method of choice. We realize, however, that this method of growing and extracting the organism is time-consuming and that the cost of obtaining specific potent antisera makes the method unacceptable for some laboratories. Laboratories not performing serological grouping should consider an alternative method based on determination of:

1. Bacitracin susceptibility.
2. Determination of the presence of the CAMP factor or hydrolysis of sodium hippurate.
3. Hydrolysis of esculin in the presence of 40% bile.
4. Tolerance to 6.5% NaCl broth.

The formulas for the media, description of the tests, and analyses of results follow. These tests, when run properly with adequate controls, will accurately identify more than 95% of the pathogenic streptococci from clinical material. The resulting identifications are, however, presumptive, and reports should indicate this fact.

B. BACITRACIN TEST

When performing the bacitracin test, do the following:

1. Use differential disks, not susceptibility disks.
2. Use a heavy inoculum.
3. Use a pure culture.
4. Use only beta-hemolytic streptococci.
5. Interpret any zone size as positive.
6. Use an infusion agar base with 5% blood.

Comments on the first five of these instructions follows.

Commercial disks that will differentiate between beta-hemolytic group A streptococci and other beta-hemolytic streptococci are available. (1) Be sure to purchase differential (0.04 units), not sensitivity disks. Disks sold and used for bacitracin sensitivity testing have too high a concentration of bacitracin to accurately differentiate between group A and non-group A streptococci. (2) A heavy inoculum of a pure culture is advisable because if the inoculum is too light, non-group A streptococci will appear to be susceptible to bacitracin. (3) Use pure cultures. Placing the differential disk on primary plates inoculated with throat swabs has shown only a 70% accuracy of identification for group A streptococci. The test is designed for use with pure cultures, not mixed cultures. (4) The test is designed for differentiating beta-hemolytic streptococci. Hemolysis must be correctly determined before this differential test can be reliable. Many alpha-hemolytic streptococci, including pneumococci, are sensitive to the bacitracin differential disk. Lots of commercial disks may vary; therefore, each new lot of disks obtained should be tested with known strains of group A and non-group A streptococci. The disks should be tested with control strains biweekly to determine the reliability of the disks. (5) The following criteria should be used to read the tests. (a) Any zone of inhibition, regardless of diameter, means that the culture is positive. (b) No zone of inhibition (growth right up to the edge of the disk) means that the culture is resistant. (c) Zone size requirements are stated in the literature, and one manufacturer, in a technical bulletin, implies that a zone size is necessary for presumptive identification of group A streptococci. The originator of the test (Maxted) did not specify that zones should be a certain size. No experimental data are available to show that zone diameters must be measured in order to differentiate group A from non-group A streptococci. False positives are potentially less harmful than false negatives. By requiring zones of 10 mm or more for presumptive identification of group A streptococci, at least one group of

investigators increased the error of the test by 10% (false negatives). The users of differential disks should realize that the bacitracin disk inhibits the growth of some strains of beta-hemolytic streptococci other than group A. Therefore, the report should b as follows: (a)"presumptive beta-hemolytic group A by bacitracin" or (b) "beta-hemolytic streptococci, not group A by bacitracin." As many as 6% of beta-hemolytic group B streptococci of human origin are susceptible to bacitracin. This error can be eliminated b using a physiological test to presumptively identify the group B streptococci. Table 8 sho the results of a recent CDC study of the use of commercial bacitracin Taxo A disks.

Note that more than 99% of the group A streptococci were susceptible to the Taxo A disks. Note also that 6% of the group B streptococcal strains were susceptible; this erro can be eliminated, however, by additional testing, which we will discuss later. The greate source of error in the bacitracin test is the susceptibility of the beta-hemolytic non-grou A, B, or D streptococci. Group C and G streptococci are occasionally (7.5%) susceptible to bacitracin and are thus erroneously presumptively identified as group A by the bacitracin test. The potential error of the bacitracin test caused by the susceptibility of the viridans streptococci (7.8%) can be eliminated by correctly identifying the hemolytic activity of the strains.

Figure 9 shows typical bacitracin and CAMP tests with group A and B streptococci. The left photograph shows a positive bacitracin test and a negative CAMP test; these responses are typical of beta-hemolytic group A streptococci. The right photograph shows a negative bacitracin test and a positive CAMP test; these responses are typical of a beta-hemolytic group B streptococci. Note particularly the heavy bacterial growth (confluent) around the bacitracin disks.

C. CAMP TEST

The CAMP test is performed by making a single streak of the streptococcus perpendicular to a streak of beta-lysin producing Staphylococcus aureus strain. Do not streak the organisms together. Note that in the photographs there is about a 1-cm space between the tw organisms. Sheep or bovine blood must be used in the blood agar plate. Washed sheep blood that has been resuspended in physiological saline and trypticase soy agar base is best. Som commercial sheep blood agar plates have been used, each lot of plates must be tested for proper reactions with control group A, B, C, and G streptococcal strains and the beta-lysin producing staphylococcus strain.

The inoculated plates should be incubated in a candle jar or in normal atmosphere. The plates for CAMP tests must not be incubated anaerobically. Some group A streptococci will be CAMP positive in the candle jar, but even more strains will be positive when the plates are incubated anaerobically. Group B streptococci produce a substance termed the CAMP factor that enhances the beta-lysin of the staphylococcus, and an area of increased lysis appears at the juncture of the two organisms. This is shown in the right part of figure 9. This area of increased lysis commonly appears in the shape of an arrowhead, as seen in Figure 9.

Bacitracin-negative, CAMP-positive, beta-hemolytic streptococci can be reported as presumptive group B streptococci. Bacitracin-positive, CAMP-positive, beta-hemolytic streptococci are either group A or group B. The bacteriologist must decide which organism the unknown strain resembles the most. This can be done by examining the hemolytic activity. Note the differences in the hemolytic activity of the two strains in Figure 9. Group B streptococci show much less surface and stab hemolysis of the blood agar than do the group A streptococci. Bacitracin-positive, CAMP-positive group A (10%) and group B (6%) streptococci occur quite frequently, thereby making it necessary for bacteriologists to familiarize themselves with the difference in their hemolytic activity.

Bacitracin-positive, CAMP-negative, beta-hemolytic streptococci are presumptive group A streptococci. Bacitracin-negative, CAMP-negative, beta-hemolytic streptococci are beta-hemolytic streptococci not group A or B.

Nonhemolytic group B streptococci are CAMP positive; therefore, nonhemolytic streptococci that are bile-esculin negative (test to be discussed later) and CAMP positive can be presumptively identified as non-hemolytic group B streptococci.

D. SODIUM HIPPURATE TEST

The following medium is used in determining the hydrolysis of sodium hippurate:

Heart Infusion Broth	25 g
Na-hippurate	10 g
Dist. H_2O	1,000 ml

Sterilize in autoclave for 15 minutes at 121° C after dispensing in 15- x 125-mm screwcap tubes. Tighten cap to prevent evaporation.

The following reagent is used in the test:

$FeCl_3 \cdot 6H_2O$	12 g
2% Aqueous HCl	100 ml

(2% Aq HCl is made by adding 5.4 ml of concentrated HCl (37%) to 94.6 ml H_2O.)

Inoculate with 2 or 3 colonies of beta-hemolytic streptococci and incubate at 35° C for 20 hours or longer, centrifuge the medium to pack the cells, and pipette 0.8 ml of the clear supernate into a Kahn tube. Add 0.2 ml of the ferric chloride reagent to the Kahn tube and mix well. If a heavy precipitate remains longer than 10 minutes, the test is positive. Immediate clearing of the tube indicates that the hippurate has not been split. Weak reactions occasionally occur, in which case the growth tube should be reincubated another 24 hours and the test repeated. Strains that yield negative reactions should be retested after an additional 24 hours growth period. Figure 10 shows the standard sodium hippurate test. Note the cloudy precipitate in the positive tube on the left; the negative tube on the right is clear.

All group B streptococci hydrolyze hippurate and therefore yield a positive test. A few group D streptococci are also positive. Other beta-hemolytic streptococci (groups A, C, G, and F) do not hydrolyze hippurate. Some alpha-hemolytic streptococci, S. uberis and S. acidominimus, hydrolyze hippurate. These strains should not interfere with the identification of the beta-hemolytic group B streptococci because of the hemolytic reaction, and they are found rarely, if at all, in human infections. S. uberis is found in bovine infections; therefore, investigators studying animal diseases, other than human, will need to rely on other characteristics to identify the isolated species. The few group D strains that hydrolyze hippurate are also BE positive; thus, strains with positive BE and hippurate reactions are presumptive group D streptococci (or enterococci, if the NaCl test is positive). The BE-negative, hippurate-positive, beta-hemolytic streptococci can be reported as "presumptive group B streptococci by hippurate hydrolysis".

Nonhemolytic group B streptococci hydrolyze hippurate; thus, they can be identified as nonhemolytic presumptive group B streptococci by hippurate hydrolysis, if the bile esculin reaction is negative.

E. OTHER PRESUMPTIVE TESTS FOR GROUP B STREPTOCOCCI

In addition to the CAMP and hippurate hydrolysis tests, a rapid hippurate test, a pigmentation test, tolerance-to-Potassium thiocynate (KCNS) test, and a peculiar reaction to glucose disks on blood agar plates have been proposed as presumptive tests for group B streptococci. We evaluated these four tests and the standard hippurate and CAMP tests in a double-blind study. These results are shown in Table 9.

The standard hippurate test most accurately identified group B streptococci. The CAMP test was the next most accurate. The only error in the CAMP test presumptive identification was with the group A strains. The difference in hemolysis was not considered when this study

was being conducted; therefore, the identification could have been improved if we had considered these differences, as previously discussed.

The glucose test was the next most accurate, but it was the most subjective and difficul test to interpret. The pigmentation test was also subjective and difficult to interpret. Th least specific tests were the KCNS tolerance test and the rapid hippurate tests.

From our experience we recommend that either the standard hippurate or CAMP tests be use to presumptively identify the group B streptococci.

F. BILE-ESCULIN (BE) TEST

Steps in making the medium used in the bile-esculin test follow:

1. Add 23 g of Nutrient agar to 400 ml H_2O, mix well, heat until colloidal.
2. Add 40 g of Oxgall to 400 ml H_2O, mix well, heat into solution.
3. Add 0.5 g of ferric citrate to 100 ml H_2O, mix well, heat into solution.
4. Combine solutions 1, 2, and 3, mix well, heat to 100° C for 10 min.
5. Sterilize into autoclave at 121° C for 15 min. (This is the base medium.)
6. Cool to 50° C.
7. Add 1 g of esculin (Aesulin to 100 ml of H_2O, heat gently to dissolve, and sterilize by filtration (Seitz or Millipore).
8. Aseptically, add sterile esculin solution to base medium, mix well, dispense into 16- x 125-mm screwcap tubes, and slant tubes.

Swan also added horse serum (50 ml) to the base medium. We, however, found, in a controlled study, that this was not necessary. In addition, at least one commercial source (Difco) has added the esculin to the base medium before sterilization. The resulting medium was just as effective as the medium described in step 8. The dehydrated medium sold by Difc can be resuspended, tubed and autoclaved, slanted, and used with excellent results.

BE medium will identify group D streptococci. All group D streptococci (includes all enterococci) will blacken the BE slant, usually within 48 hours. Most non-group D streptococci do not blacken the medium. Figure 11 shows the bile esculin test; the tube on the right is blackened (positive), and no reaction has occurred in the tube on the left (negative). Not all group D streptococci are enterococci (penicillin resistant). S. bovis and S. equinus are group D species, but they are not enterococci. S. bovis (BE positive) is found in a significant number of group D infections and is penicillin sensitive; therefore, further tests are needed to adequately differentiate the enterococci from nonenterococci. A streptococcus that gives a positive BE reaction should be reported as a "presumptive group D streptococcus by BE hydrolysis". The BE test does not differentiate enterococci from nonenterococci; thus a positive BE test should not be reported as presumptive identification of enterococci.

Table 10 shows the data from two studies. Several presumptive tests used to identify group D streptococci were evaluated. The modified BE medium is the same as BE medium except that the horse serum was omitted from the formula. The same strains that were tested on the BE and modified BE were tested on Pfizer Selective Enterococcus (PSE) Agar. More viridans streptococci gave positive reactions on PSE than on BE or modified BE media. Eosin Methylene Blue (EMB) Agar and methylene blue milk (MBM) showed nonspecificity (12% and 13% positive reactions with viridans streptococci after 48 hours) and less sensitivity than BE, modified BE, and PSE. Only 89% of the group D strains were positive on either EMB or MBM.

G. SALT TOLERANCE TEST

The formula for determining tolerance to 6.5% NaCl is as follows:

Heart Infusion Broth	25 g
NaCl	60 g
Dextrose	1 g

Indicator (1.6 g brom cresol
purple in 100 ml ethanol) 1 ml

Dist. H_2O 1,000 ml

Mix reagents, dissolve, mix, dispense in suitable tubes (15- x 125-mm screwcap), and autoclave at 15 lbs pressure for 15 minutes.

Enterococci (S. faecalis and its varieties zymogenes and liquefaciens, S. faecium and S. durans) will usually give heavy growth and an indicator change within 24 hours. Some enterococcal strains take 48 hours, and some will grow with no accompanying indicator change even after 72 hours. About 80% of group B streptococci will also grow in this medium, and some change the indicator. Beta group A, C, G, and F usually do not grow in the medium. The alpha-hemolytic, non-groupable streptococci (viridans), such as S. mitis, S. sanguis, S. salivarius, S. mutans and S. MG, do not grow in 56.5% NaCl medium, nor do group D species S. bovis and S. equinus.

Figure 12 shows the salt tolerance test. The positive tube on the left is cloudy with growth; the negative tube on the right is clear. Note especially the heavy growth at the bottom of the left tube (positive). It is typical of enterococcal growth.

A positive BE test and positive growth in 6.5% NaCl broth confirms the presence of enterococci. Even if serological reactions are determined, growth in 6.5% NaCl broth should be used to confirm that the group D streptococcus is an enterococcus. Salt-tolerant, beta-hemolytic strains other than groups B and D streptococci occur very rarely. Salt-tolerant group streptococci occur occasionally. Strains that give positive salt tolerance tests and that are a presumptive group B (hippurate) or presumptive group D (BE) streptococci should be tested for purity by streaking the growth from the salt-tolerance-test medium onto a blood group plate and comparing the morphology to that of the original strain. If the morphology differs a Gram stain and a catalase test should be performed.

Table 11 shows the data from two studies in which several tests to be used with the BE test for identification of enterococci were evaluated. Ideally, only the enterococcal group D strains and no other streptococcal strains should grow in any of the tests. Note in Table 11 that the enterococcal strains grow well in all media; however, note the variability of growth of the group D nonenterococci and viridans streptococci in the two different lots of Streptococcus faecalis (SF) broth. Lot X was very effective in differentiating the enterococcal from nonenterococcal strains, but lot Y was very ineffective in the same differentiation. The variability from lot to lot implies that growth in SF broth is not a reliable test for identification of enterococci. KF broth was also nonspecific; many viridans strains were capable of growing in KF broth.

These results show that growth in either modified or unmodified 6.5% broth is a more reliable indicator of enterococci than growth in other broths tested. The fact that some beta-hemolytic streptococci (Group A, B, C, F, and G) may grow in 6.5% NaCl broth indicates that the growth in 6.5% NaCl broth cannot be used by itself as a single criteria for identifying enterococci. Only enterococci are BE and NaCl positive. Group D nonenterococcal streptococci (S. bovis) are BE positive and NaCl negative, and the viridans streptococci are negative in both BE and NaCl tests. All beta-hemolytic group A, B, C, F and G streptococci are BE negative.

E. IDENTIFICATION OF PNEUMOCOCCI

Since the genus Streptococcus includes the pneumococci as part of the new classification, two tests that can be used to presumptively identify Streptococcus pneumoniae should be mentioned.

1. Optochin test

Figure 13 shows an optochin (ethylhydrocupreine) test. Three or four suspect colonies are streaked on a quarter plate, and the optochin disk is placed in the upper third of the

streaked area. The plate is incubated in a candle jar or CO_2 incubator. Normal atmospheric incubation diminishes the growth, and larger zones of inhibition occur. Some strains of pneumococci require increased CO_2 for growth. Since this is not a standardized test, with standard inoculum, agar media, or quality controlled disks it should be used for presumptive identification. There are requirements for diameters of zones of inhibition, but a wide variation in the critical zone should be allowed. That is, if a 6-mm disk is used, the inhibition zone should have a diameter of at least 14 mm for the test to be considered positive for pneumococci. If the diameter is between 6 and 14 mm, the result is questionable, and the strain should be tested for bile solubility. Strains that are bile soluble and give small zones of optochin sensitivity should be identified as pneumococci. If the larger 10-mm optochin disk is used, an inhibition zone of 16 mm in diameter or greater should be required for presumptive identification, and strains with inhibition zones between 10 and 16 mm should be tested for bile solubility.

Table 12 shows the data that support these suggestions. Note that if a zone size of 16 mm is required for presumptive pneumococcal identification, 6.6% of the strains would not be properly identified with the 6-mm disk. If the zone size requirement is lowered to 14 mm, only 1.6% of the strains would not be properly identified. Small zones of inhibition cannot be interpreted as presumptive identification of pneumococci because some viridans streptococci will yield inhibition zones in the questionable range (10 to 14 mm). The larger disks (10 mm) show less error than do the smaller disks; however, more viridans streptococci will show small zones of inhibition with the larger disks than with the smaller disks.

2. Bile solubility tests

Figure 14 shows a bile solubility test. Half of a millilitre of a neutralized broth culture is placed in each of two 13- x 100-mm test tubes. Phenol red indicator and normal NaOH is used to adjust the broth to pH 7.0.

Add half a ml of 2% Na desoxycholate to one tube and half a ml of saline to the other. Place at 37° C and examine periodically for up to 2 hours. A clearing of turbidity indicates a positive test: that is, the pneumococcal cells have been lysed or solubilized. Note that the tube on the left in Figure 14 is completely cleared; this is the tube with 2% sodium desoxycholate. The tube on the right is cloudy; this is the saline control. All pneumococci are bile soluble, but some only partially solubilize in the test.

Therefore, partially soluble strains that are susceptible to optochin should be identified as pneumococci. Partially soluble strains that are resistant to optochin are not pneumococci and should be identified as alpha-streptococci, not as pneumococci.

We have based our opinions that partially soluble strains should be tested for optochin susceptibility on the results in Table 13. We investigated both 10% and 2% sodium desoxycholate for effectiveness in solubilizing pneumococci. First of all, results of the 10% and 2% solubility tests were in 93.3% agreement. Second, 86% of the strains were completely soluble in 10% sodium desoxycholate, and 83% were completely soluble in 2% sodium desoxycholate. These results indicate that either 10% or 2% sodium desoxycholate can be used in the bile solublity test and that a second test (optochin susceptibility) is needed to bring the presumptive identification of pneumococci above 95%.

I. SUMMARY AND TABLE OF PRESUMPTIVE TEST IDENTIFICATION OF STREPTOCOCCI

All of the tests that we have discussed for presumptive identification are summarized in Table 14. Remember particularly that determining hemolytic activity is the most important step in the presumptive identification of streptococci.

The first column identifies group A streptococci. They are beta-hemolytic, susceptible to bacitracin, do not hydrolyze hippurate, do not give a positive CAMP reaction, and do not hydrolyze BE or grow in 6.5% NaCl broth.

The second column identifies group B streptococci; they are beta-hemolytic and are usually resistant to bacitracin, although occasional strains are susceptible to bacitracin. They hydrolyze hippurate and give a positive CAMP reaction. They DO NOT hydrolyze bile

esculin medium, and some strains tolerate 6.5% NaCl broth, especially after 48 hours' incubation.

The third column identifies the beta-hemolytic streptococci that are not group A, B, or D streptococci. They are beta-hemolytic strains that are resistant to bacitracin, do not hydrolyze hippurate or BE, do not give a positive CAMP reaction, and usually do not tolerate 6.5% NaCl broth. They are identified as "beta-hemolytic streptococci not group A, B, or D". Group C, G, and F streptococci fall into this category.

The fourth column identifies the group D enterococcal streptococci; enterococci are BE and 6.5% NaCl positive. An occasional strain will hydrolyze hippurate, but any strain that is BE positive is a group D streptococcus, and if it tolerates 6.5% NaCl, it is an enterococcus. These strains can be alpha, beta, or nonhemolytic. They do not give positive CAMP reactions.

The fifth column identifies the group D nonenterococcal streptococci. They produce positive results only in the BE test. These strains do not tolerate 6.5% NaCl, or hydrolyze hippurate or give positive CAMP reactions, and they are usually nonhemolytic. They are susceptible to penicillin, whereas the enterococci are resistant. Patients with nonenterococcal group D infections can be managed as if they had a viridans streptococcal infecton, but patients with systemic enterococcal infections must be treated with combined antibiotic therapy if the causative organisms are to be eradicated.

The sixth column identifies the viridans streptococci. They are alpha-hemolytic or nonhemolytic strains that do not hydrolyze bile esculin or hippurate, do not give positive CAMP reactions, and do not grow in 6.5% NaCl broth. Some of the strains are susceptible to bacitracin, but they are still viridans streptococci. Occasional alpha or nonhemolytic group B streptococci can be presumptively identified as such because they hydrolyze hippurate and give positive CAMP reactions and do not react in bile esculin tests. This identification should be confirmed by serogrouping.

The last column identifies the pneumococci. About 50% of the pneumococci are susceptible to bacitracin, and this fact indicates the need to correctly determine hemolysis. For convenience, the hippurate hydrolysis, BE, and sodium chloride tolerance tests are noted here; however, CDC does not advocate using these tests for suspect pneumococcal colonies.

These presumptive tests, when performed properly, will place approximately 85% of the streptococci in the groupings listed in Table 14.

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PART IV

AGGLUTINATION GROUPING

A. INTRODUCTION

In Part II of this manual we state that the best method for identifying the beta-hemolytic streptococci *is to extract the carbohydrate antigen from* the streptococci and react the antigen extract with group specific precipitating antisera. This procedure requires growing the streptococci in pure culture overnight and then extracting the antigen to react with group specific antisera. Recently, two slide agglutination tests that utilize carrier particles for the group-specific antisera have been described. These tests require 24 hours less than conventional grouping procedures. They are the Phadebact Streptococcus test (1), manufactured by Pharmacia Diagnostics, and the Streptex test (1) manufactured by Wellcome Reagents Limited. The reagents for these tests are now commercially available.

The Phadebact test uses specifically prepared protein A-rich staphylococcal cells conjugated to group-specific streptococcal antisera. The test is called the coagglutination (CA) test. The Streptex test uses latex particles conjugated to group-specific streptococcal antisera. This test is called the latex agglutination (LA) test. The carrier particles in these tests (staphylococcal cells and latex particles) are so large that a positive agglutination can be seen without the aid of magnification. Positive CA and LA reactions look very much alike, and so do the negative reactions. Figure 15 depicts typical reactions for either test. Note the clumping in the reaction on the left. This is a positive reaction. Note the smooth, milky appearance of the reaction on the right. It is negative.

B. COAGGLUTINATION (CA) TEST

The CA test is designed to identify group A, B, C, and G beta-hemolytic streptococci. Group D and other nonbeta-hemolytic streptococci should not be tested with the CA reagents because erroneous identification may result.

Pure cultures of streptococci should be used because other bacteria may react with the CA reagents and erroneous identification may result.

To avoid possble "broth reactions" the manufacturer suggests that Bioquest Todd-Hewitt broth (THB) be used to prepare the antigens. The broth reaction <u>may</u> occur when the same broth is used to prepare the antigen for the CA test that was used to prepare the streptococcal group specific antisera. When streptococcal grouping antisera are prepared, the animals immunized with streptococcal cells may form antibodies to the broth in which the cells were grown. These antibodies are not detected by the usual capillary precipitin test but may be detected by the CA test because it is much more sensitive. Current policies are to prepare the CA reagents from streptococcal antisera prepared for the capillary or ring precipitin tests. Thus, exceptional care should be taken to ensure that the broth reaction is eliminated as a possible source of error. A good quality control measure is to test each lot of uninoculated broth with each new lot of CA reagents.

1. *Methods for preparing the antigens*

(a) Overnight broth suspensions
- (1) Inoculate 2 ml of broth with one or two colonies of beta-hemolytic streptococci.
- (2) Incubate overnight at 35° C - 37° C.
- (3) Mix suspension thoroughly by vortexing or vigorous rotation.

(b) Four-hour broth suspensions
- (1) Inoculate 2 ml of broth with several (four or more) beta-hemolytic colonies.
- (2) Incubate for 4 h at 35° C - 37° C.
- (3) Mix suspension thoroughly by vortexing or vigorous rotation.

(c) Direct testing

Transfer at least five beta-hemolytic streptococcal colonies directly to each of the four CA reagents (see Section D.2.d. of this manual).

(d) Overnight broth supernatants

(1) Inoculate 2 ml of broth with one beta-hemolytic colony.

(2) Incubate overnight at 35° C - 37° C.

(3) Centrifuge to sediment the cells.

2. Performance of the test

(a) Label a clean, dry glass slide (2 x 3 in. or larger) with the letters A, B, C, and G. The distance between the labels should be at least 20 mm. If 1- x 3-in. slides are used only two CA reagents per slide should be tested. The volume of the reagents is such that they will run together or off the slide (contaminating your fingers) if the reactants are not placed a sufficient distance apart.

(b) Put one full drop of each of the four reagents on the appropriately labeled slide(s). Because the conjugated cells tend to settle out of solution, shake each reagent well to mix the contents before transferring a drop to the slide.

(c) Add one full drop of the antigen to each of the four reagents on the slide(s). The antigen preparations are described in 1.a., 1.b., and 1.d above.

(d) Mix the antigen and CA reagents with an applicator stick using a separate stick for each reagent.

(e) Rock the slide to and fro gently for about 1 min or until a positive reaction occurs

(f) Watch for agglutination by using transillumination against a dark background. Usually, only one reagent will give positive agglutination with the cell suspensions; in some cases, however, more than one reagent will show a positive reaction. Under these circumstances, the fastest and strongest reaction is recorded as the correct reaction. When two or more reagents react equally, the test must be repeated with a new antigen preparation or a trypsin treated antigen preparation.

3. Trypsin-treated antigens

This procedure is used to modify cell suspensions used as antigens (4-hour and overnight cell suspensions). Cell suspensions that react with more than one CA reagent can, in most cases be made to react with only one CA reagent by treating the cell suspension with trypsin. Trypsin is a proteolytic enzyme that hydrolyzes proteins on the outer surface of the streptococcal cells. These protein antigens cause most of the cross-reactions in the CA test.

(a) Prepare a 5% trypsin solution (Difco 1:250) by adding 5 g of trypsin powder to 100 ml of distilled water. Mix for 2 hours on a magnetic stirrer at 4° C. Steralize by filtration. This solution can be stored at 4° C for as long as 6 months. However, as much as 75% of its activity is lost if the solution is left at room temperature for 3 hours.

(b) Add two drops of sterile 5% trypsin to the cell suspension.

(c) Adjust pH of suspension to about 8.2 with N/5 NaOH.

(d) Incubate at 35° C - 37° C for 30-60 minutes.

(e) Retest as described in 2. above.

4. Results

(a) Overnight broth suspensions used as antigens.

Table 15 shows the results we obtained when overnight broth suspensions were used as antigens. These results were obtained when the streptococci were grown in Difco THB. It was during these experiments that we discovered the broth reaction: that is, we tested

suspensions of cultures grown in the same broth in which streptococcal cells used for antisera preparation were grown. This is why so many of the antigen suspensions gave multiple reactions. Note the results shown in Table 15; only half of the group A strains were correctly identified when first tested. Eleven of the 24 strains gave multiple reactions. Note also that most of the streptococci that gave multiple reactions belong to group A, C, or G. These streptococci share protein antigens, and are more likely to cross react with each other's antiserum than with the group B antiserum. The group B and F streptococci only occasionally share these same antigens and therefore do not cross-react with the other streptococcal antisera.

Note that suspension of two of the eight group D streptococci tested were erroneously identified, one as a group A, and the other as a group B streptococcus.

The data shown in part B of Table 15 indicated that not all of the problems associated with suspensions that gave more than one reaction in the CA test were resolved. As stated earlier, the multiple reactions were probably due to the broth used. Other investigators have obtained much better results when broth other than Difco THB was used to prepare the antigens. We give these data here only to emphasize the kinds of problems that may be encountered if instructions are not properly followed.

(b) Four-hour broth suspensions used as antigens.

Table 16 shows the results we obtained when 4-hour broth suspensions were reacted with the CA reagents. We used BBL-THB in these experiments and made two important findings. First, we found that some of the cell suspensions of group A, C, and G streptococci still gave multiple reactions with the CA reagents. However, all of the multiple reactions were resolved with the trypsin technique. This is shown in Part B of Table 16. Second, we found that 3 of the 7 suspensions of group D streptococci were erroneously identified - one as a group A, another as a group C, and a third as a group G streptococcus. A procedure to keep group D streptococci from being tested with these reagents must be used to avoid erroneous identification. We suggest such a procedure later.

(c) Overnight supernatants used as antigens.

Table 17 shows the results we obtained when overnight broth supernatants (BBL-THB) were used as antigens. The reactions observed with this antigen preparation were the easiest to read. No multiple reactions were found. All of the group A, B, C, E, F, and G streptococci were correctly identified. The only errors were with two group D strains, a problem we have already brought to your attention.

(d) Other antigen preparations.

We have received published and unpublished reports of testing 4-hour broth supernatants with the CA reagents, and these reports have been favourable. We have had unfavourable reports from investigators attempting the direct test, that is, testing colonies taken directly from an agar plate and mixing them with the CA reagents. We have had no experience with either of these procedures and are therefore reluctant to make recommendations at this time.

A third alternative procedure has been described, but only for group B streptococci. In this procedure swabs were placed into selective broth medium made up to inhibit bacteria other than group B streptococci. In fact, some strains of streptococci are also inhibited. The broth was incubated overnight at 35° C and after being mixed was tested for reaction with CA reagents. More than 98% of the group B streptococci were correctly identified. This procedure has not been tested for group A streptococcal identification and should not be used for this purpose at this time. If experiments similar to those described for group B streptococci are successful for group A, however, this technique may become very useful for identifying group A streptococci.

C. LATEX AGGLUTINATION (LA) TEST

The LA test includes reagents to identify group A, B, C, D, F, an G streptococci. Since the kit contains a reagent for identifying group D streptococci and since most group D streptococci are nonbeta-heymolytic, this kit can be used to identify nonbeta- as well as beta-hemolytic streptococci.

Although we have not tested mixed cultures, the manufacturer states that the cultures need not be pure for the test to accurately identify the group antigen of the streptococci. Our results indicate that when sufficient quantities of beta-hemolytic colonies are present, the LA test is very accurate.

Because the antigen preparations for the LA tests are extracts, broth cross-reactions are not a problem. Because extracts of cellular growth of streptococci are used, the LA reagents are not in immediate contact with the culture medium, thus eliminating this possible source of error. This does not, however, eliminate the need for good quality control practices. Each lot of LA reagents should be tested for correct reactions with extracts of stock strains.

1. Methods for preparing the antigens

(a) Pronase extraction of blood agar plate growth.

(1) Resuspend pronase solution provided with the Streptex Kit according to the manufacturer's instructions.
(2) Put 0.4 ml pronase solution in a 12- x 75-mm tube.
(3) Make a sweep through confluent growth on a blood agar plate with a bacteriological loop and transfer to the pronase solution. Repeat two more times. The pronase-cell mixture should be milky; if not, transfer additional growth from the plate to the pronase solution. Most of the growth transferred should be streptococcal cells but other bacterial cells will not interfere with the results.
(4) Incubate in a 56° C hot water bath for 60 minutes.
(5) Remove from water bath and sediment cells by centrifugation. The clear supernatant is the extract.

(b) Modified autoclave extract of blood agar plate growth.

(1) Put 0.2 ml of physiological saline in a 5- x 50-mm Durham fermentation tube.
(2) Make three sweeps through confluent growth on a blood agar plate with bacteriological loop and transfer each loopful to the saline solution. The saline cell mixture should be milky; if it is not, transfer additional growth from the plate to the saline solution.
(3) Put nonabsorbant cotton plug in open end of Durham tube.
(4) Autoclave the mixture (121° C for 15 minutes).
(5) Remove from autoclave, remove cotton plug, and sediment the cells by centrifugation. The clear supernatant is the extract.

(c) Pronase extract of overnight broth culture.

(1) Inoculate 20 ml of THB with one or two colonies of streptococci.
(2) Incubate culture overnight at 35° C - 37°C.
(3) Sediment cells by centrifugation.
(4) Decant broth and save sedimented cells.
(5) Add 0.4 ml of pronase solution to sedimented cells and mix to suspend cells in pronase.
(6) Place in 56° C hot water bath for 60 minutes.
(7) Remove from hot water bath and sediment cells by centrifugation. The clear supernatant is the extract.

2. Performance of the test

(a) Using a Pasteur pipette, transfer one drop of the extract into each of the six circles on the glass tile provided with the kit. Half drops of the modified autoclave extracts can be used.

(b) Place one drop of each LA reagent in the appropriately labeled circle. Be sure to mix each reagent by shaking the vial before transferring reagents to the tile. Half-drops of each reagent can be used when testing the modified autoclave extracts.

(c) Mix the contents of each circle with a separate applicator stick.

(d) Rock the slide gently to and fro for 2 minutes or until a positive reaction is observed.

(e) Observe the reaction with reflected light. The back of the tile is coated with a black substance. Reactions are easily observed 25-30 mm from the eye.

3. Results

(a) Pronase and modified autoclave extracts of beta-hemolytic streptoccoci from blood agar plates.

Table 18 shows the results of testing both the pronase and modified autoclave extracts made from blood agar plate growth of 155 strains of beta-hemolytic streptococci. The LA reagents correctly identified all the strains of group A, B, C, F, and G streptococci with both extraction procedures. The only disagreement between results of conventional grouping procedures (capillary precipitin testing of Lancefield extracts) and the LA tests were found among beta-hemolytic strains of streptococci whose conventional extracts did not react with CDC grouping antisera in the capillary precipitin test. This is shown in the next to the last line in Table 18. Pronase extracts of four and autoclave extract of one of these strains reacted with the LA group F reagent. The beta-hemolytic nongroupable streptococci used in this study were similar to the beta-hemolytic group F streptococci in every respect except that a group antigen could not be demonstrated for them by conventional techniques. The LA test is probably more sensitive than the conventional procedures, and the group F reactions observed with the LA reagents and these strains are probably correct. We concluded that the LA reagents identified 100% of the beta-hemolytic streptococci when extracted by either pronase or modified autoclave techniques.

(b) Pronase and modified extracts of nonbeta-hemolytic streptococci from blood agar plates.

Table 19 shows the LA reactions with both pronase and modified autoclave extracts made from streptococcal growth on blood agar plates. First, by comparing the results in columns 3 and 4 to those columns 5 and 6, note that there is very little difference in results obtained with the pronase and modified autoclave extracts. The differences are not important in the final identity of the strains. Second, note that all three nonhemolytic group B strains were correctly identified. Third, note that all of the group D S. faecalis and four of five of the group D S. faecium strains were correctly identified as group D streptococci. Conventional Lancefield extracts of the one S. faecium strain that was not identified as a group D streptococcus by the LA procedures did not give a positive capillary precipitin reaction. Thus, we were unable to demostrate that this strain possessed a group D antigen by conventional techniques.

Observe that only 3 of 13 strains of S. bovis were correctly identified as group D streptococci by the LA reagents with either pronase or modified autoclave extracts. This was the largest error in the LA test. All 13 strains of S. bovis were confirmed as group D streptococci by conventional Lancefield extraction and capillary precipitin tests. We describe a procedure that will minimize these errors on page . The latex agglutination reactions listed on the next four lines, that is C, F, G, and None viridans, are not important for the identity of the viridans strains. The reasons for this are explained in Part II, section A.2. and Table 3 of this manual.

(c) Pronase extracts from overnight broth cultures

Table 20 shows the results of LA testing of pronase extracts made from both beta- and nonbeta-hemolytic streptococci grown in 20 ml THB cultures. First note that all of the beta-hemolytic group A, B, C, F, and G streptococci were correctly identified. Second, no that 13 of the 15 group D strains tested were correctly identified. Included in the 15 gr D strains tested were 12 strains of S. bovis. Only 2 of these 12 strains were not identif as group D streptococci by the latex group D reagent. Third, and most important, we would like to point out what happened when the nonbeta-hemolytic streptococci were extracted fro 20-ml THB cultures. This is found on the last three lines of Table 20. The viridans stra tested are listed as nonbeta-hemolytic group C, F, and none. Note that 8 of the 16 strain tested were identified as group D streptococci by the LA group D reagent. Conventional Lancefield extraction and capillary precipitin testing of these strains demonstrated that none of these strains possessed the group D antigen. Group C reactions were obtained from of these strains by conventional techniques, but only one group C reaction was observed wit the LA technique. The one strain that was identified as having the group C antigen by both conventional and LA procedures was proven to possess the group C antigen by double gel diffusion. We were unable to confirm the group C reactions as valid by double gel diffusic of the other six strains.

We concluded from these experiments that the CDC group C precipitating and the LA group antisera have a common cross-reacting antibody. This is not unexpected, since during the preparation of streptococcal grouping antisera the antisera are not tested for cross-reactions with extracts of the viridans species. We conclude that the pronase extraction LA procedure from 20 ml of broth is satisfactory for beta-hemolytic streptococci but unsatisfactory for nonbeta-hemolytic streptococci.

D. SUMMARY AND RECOMMENDATIONS

1. Techniques for confirmation of group D streptococci

Because both the CA and LA tests present the potential for serious errors in identifying group D streptococci, we recommend a battery of two physiological tests to aid in this identification. In addition, since enterococcal and nonenterococcal group D streptococci differ in susceptibility to antibiotics, microbiologists must have a means of differentiatir them. We recommend testing all suspect group D streptococci for bile esculin (BE) reactions and tolerance to 6.5% NaCl broth. The formulas for the media are described in Part III, sections F and G of this manual. To decrease the number of BE and NaCl tests to perform, we suggest that microbiologists do not need to determine these reactions if the culture is from the oropharynx. Group D streptococci are not commonly found in throat swabs and if they are they are of little significance. If sheep blood agar is used for determining streptococcal hemolysis, most group D streptococci will be nonbeta-hemolytic. Thus, the use of sheep bloo for processing throat swabs would eliminate most group D strains from being tested for group reactions. Nonbeta-hemolytic streptococci are considered normal oral flora. If the source of the culture is from other body sites or fluids however, the BE and NaCl tests must be performed to minimize the potential errors in the CA and LA procedures.

2. Recommendations for CA testing

(a) Test only beta-hemolytic strains of streptococci. The manufacturer recommends this and we concur. CA reactions of nonbeta-hemolytic strains are not valid.

(b) Use BBL-THB, which the manufacturer recommends, for preparing broth suspensions and supernatants. We have also tested trypticase soy broth for preparing 4-hour broth suspensions, overnight suspensions, and overnight supernatants. All of these preparations have been satisfactory (unpublished data). Specifically, however, avoid using DIFCO THB.

(c) Use a method, such as the one described in D.1 of this manual, for identifying group D streptococci. This will eliminate erroneous identification of group D streptococci as group A, B, C, or G by the CA test.

(d) Prepare the antigen for the CA test from 4-hour broth suspensions. This is the most convenient method. Overnight broth suspensions are accurate but less convenient. Overnight broth supernatants are the most accurate; they can be used to confirm or resolve reactions of either 4-hour or overnight suspensions. Direct testing of agar plate growth with CA reagents is not a proven useful technique. We do not recommend it, but future experiments may resolve some of the problems.

(e) Prepare a 5% solution of trypsin to treat cell suspension-antigens that react with more than one CA reagent. The manufacturer does not recommend this procedure, but we found it very useful.

3. Recommendations for LA testing

(a) Use LA reagents to identify both beta and nonbeta-hemolytic streptococci. The LA reagents are very accurate for identifying beta-hemolytic strains and are conditionally accurate for identifying nonbeta-hemolytic strains.

(b) Do not use trypsin. It is not needed because multiple reactions are not observed with the CA reagents.

(c) Use pronase or modified autoclave extracts of growth taken from blood agar plates. Both procedures are accurate and require about the same amount of time to perform. The autoclave technique is more economical because it requires only half the volume of LA reagents.

(d) Use the pronase extraction - LA of overnight broth cultures to identify the group antigen of the beta-hemolytic streptococci, but do not use this procedure to identify the group antigens of nonbeta-hemolytic streptococci.

(e) Test nonbeta-hemolytic streptococci for BE and NCl reactions to aid the identification of group D streptococci, because of the failure of LA reagents to detect the group D antigen from S. bovis strains in both pronase and modified autoclave extracts of agar plate growth.

4. Additional comments

Finally, we would like to advise you of three things. First both the CA an LA reagents are more expensive than those used for conventional grouping techniques. Generally, the CA and LA procedures are more convenient, and usually when they are used instead of conventional procedures, one full day is saved in the identification process. Therefore, you will need to choose between cost and convenience. Second the maufacturer's instructions or those described in this manual should be followed. Generally, the instructions are similar, and we have attempted to define the limits of each test. Third, because of the enhanced sensitivity of these tests, the specifity of new lots of reagent used in the tests may vary widely. A quality control program should be developed for testing each new lot as it is purchased. We hope that these reagents will be improved to the point that cross-reactions are eliminated and specificity enhanced.

E. REFERENCES

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2. Latex agglutination

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Table 1. Transport Methods for Streptococcal Swabs

Transit Time	*Transport System*
0-2 hours	None
2-24 hours	Transport media (Stuart's or Amies')
1-several days	Silica gel

Table 2. Serological reactions of beta-hemolytic streptococci of human origin identified from 1969 through 1975.

Group	Number	Percent of total
A	9,587	67
B	3,444	24
C	435	3
F	133	1
G	535	4
A-var, A-int, L	3	<1
none	80	1

Over the same period of time 98% of 665 group D strains were reactive with group D antiserum.

Table 3. Serological cross-reactions of 1187 viridans streptococci of human origin.

Species	Serological group A	B	C	D	F	G	Number tested
Uberis	0	0	0	0	0	0	7
Mutans	0	0	0	0	3	0	152
Sanguis I	0	0	4	0	22	0	202
Salivarius	0	0	0	0	3	0	81
MG-intermedius	9	0	6	0	53	3	231
Sanguis II	6	0	6	0	4	2	231
Mitis	4	0	4	0	2	2	177
Anginosus-constellatus	2	0	1	0	8	2	55
Morbillorium	0	0	0	0	0	0	46
Acidominimus	0	0	0	0	1	0	5
Total number of cross-reactions	21	0	21	0	96	9	147
% of total	1.8	-	1.8	-	8.1	0.8	12.4

Table 4. Serological classification of streptococci.

Hemolysis	Group	Species
Beta	A	S. pyogenes
Beta	B	S. agalactiae
Beta	C	S. equisimilis
Beta	C	S. zooepidemicus
Beta	C	S. equi
Alpha, beta, none	D	S. faecalis
Alpha, none	D	S. faecium. S. durans
Alpha, none	D	S. bovis, S. equinus
Beta	F	S. anginosus
Beta	G	No species name

Table 5. Differentiation of group C and D streptococci

A. Group C strains	Physiological tests	
	Trehalose	Sorbitol
S. equisimilis	+	-
S. zooepidemicus	-	+
S. equi	-	-

B. Group D strains	6.5% NaCl	Arginine	Pyruvate
S. faecalis	+	+	+
S. faecium	+	+	-
S. bovis	-	-	-

Table 6. Extraction techniques for serological grouping of streptococci.

Author	Technique	Complexity
Lancefield	hot-acid	two pH adjustments
Fuller	hot-formamide	one pH adjustment
Rantz	autoclave	none
El Kholy	nitrous acid	one pH adjustment
Maxted	*S. albus* enzyme	none
Ederer	Pronase B enzyme	none
Watson	*S. albus*-lysozyme enzymes	none

Table 7. MODIFICATIONS OF THE FLUORESCENT* ANTIBODY STAINING TECHNIQUE FOR IDENTIFICATION OF GROUP A STREPTOCOCCI.

Author	Modification (staining time and temp.)
Moody, original method	none—30 minutes at room temperature.
Martin	1 minute at 45 C.
Freeburg	trypsinization plus 30 seconds at 50 C.
Ederer	1 to 2 minutes at 50 C.

Moody, Martin and Freeburg used broth cultures, and Ederer used blood agar plate cultures for specimens.

Table 8. Bacitracin Susceptibility of 1,132 Streptococcal Strains When Tested With Commercial Bacitracin Disks.

Streptococci	No. tested	Percent positive
Group A	571	99.5
Group B	184	6.0
Beta-Strep, non A, B, or D	134	7.5
Group D	90	1.1
Viridans	153	7.8

Table 9. Percent Positive Reactions of Various Streptococci in Six Presumptive Tests Designed to Identify Group B streptococci.

Group	Number of Strains	Std hip	Rap hip	CAMP	Pigment	KCNS tol	Glucose
A	30	3	3	10	0	10	0
B	40	100	68	98	85	85	93
C	31	0	0	0	0	16	0
G	30	0	3	0	0	40	0
F	1	0	0	0	0	0	0
D	7	0	29	0	29	100	86
Vir.	4	0	0	0	0	0	25

Std hip = Standard hippurate, Rap hip = Rapid hippurate, KCNS tol = Tolerance to potassium thiocyanate.

Table 10. Percent Positive Reactions of Various Streptococci in Five Presumptive Tests Designed to Identify Group D Streptococci.

Streptococci	Bile-esculin		Modified bile esculin		Pfizer Selective Enterococcus Agar		Eosin Methylene blue		Methylene blue milk	
	24	48	24	48	24	48	24	48	24	48
beta-hemolytic group A, B, C, F, and G	0	0	0	0	1	5	ND	0	5	23
Group D includes enterococcal and non-enterococcal strains	96	100	97	100	98	100	ND	89	85	89
viridans	1	4	1	3	9	24	ND	12	3	13

ND = Not Done

Table 11. Test Results for Enterococcal Identification

	6.5% NaCl broth		Modified 6.5% NaCl broth		SF broth lot X		SF broth lot Y		KF broth	
Streptococci	24	48	24	48	24	48	24	48	24	48
Beta-hemolytic group A, B, C, F and G	11	15	17	19	0	0	ND	ND	ND	40
Group D enterococci	97	97	96	99	91	99	100	ND	ND	99
Group D nonenterococci	0	0	0	0	4	4	54	ND	ND	99
Viridans	0	2	0	0	1	1	31	ND	ND	42

ND = Not Done

Table 12. Comparison of Diameter of Zones of Inhibition (mm) of *S. pneumoniae* with Two Different Optochin Disks

Diameter of inhibition zones	BBL optochin (7 mm disk)	Difco optochin (10-mm disk)
less than 16 mm	24 (6.6%)	7 (1.9%)
equal to or greater than 16 mm	338 (93.4%)	355 (98.1%)
less than 14 mm	9 (2.6%)	3 (0.8%)
equal to or greater than	353 (97.4%)	359 (99.2%)

Table 13. Comparison of Two Modifications of the Bile Solubility Test with 263 Pneumococcal Strains

Solubility in: 10% Sodium desoxycholate	2% Sodium desoxycholate	No. strains	percent
positive	positive	216	82.1
partial	partial	23	8.7
negative	negative	8	3.0
positive	partial	7	2.7
positive	negative	4	1.5
partial	positive	4	1.5
negative	partial	1	0.4

Table 14. Presumptive Identification of Streptococci based on Physiological Findings.

Hemolysis	Beta	Beta	Beta	Alpha beta none	Alpha none	Alpha none	Alpha
Bacitricin susceptibility	+	-*	-*	-	-	-*	±
Hippurate hydrolysis or CAMP reaction	-	+	-	-	-	-*	-
Bile esculin hydrolysis	-	-	-	+	+	-	-
Tolerance to 6.5% NACl	-	±	-	+	-	-	-
Optochin susceptibility or bile solubility	-	-	-	-	-	-	+
Presumptive identification	Group A	Group B	Beta hemo-lytic not group A, B, or D	Group D entero-cocci	Group D not an entero-coccus	Viri-dans	Pneumo-cocci

Table 15. Reactions of *Overnight Broth Suspensions* of Beta-Hemolytic Streptococci with Coagglutination Grouping Reagents

Part A. Reactions without trypsin

Strains		Phadebact reagent				No Reaction	Multiple Reactions
Group	Number	A	B	C	G		
A	24	12	0	0	0	1	11
B	23	0	23	0	0	0	0
C	15	0	0	10	0	1	4
D	8	1	1	0	0	4	2
E	2	0	0	0	0	2	0
F	7	0	0	0	0	7	0
G	19	0	0	0	6	0	13

Part B. Reactions after trypsinization

Group	Number	A	B	C	G	No Reaction	Multiple Reactions
A	11	8	0	0	0	0	3
C	4	0	0	4	0	0	0
D	2	0	0	0	0	0	2
G	13	0	0	0	2	0	11

Table 16. Reactions of *4 hour Broth Suspensions* of Beta-Hemolytic Streptococci with Coagglutination Grouping Reagents

Part A. Reactions without trypsin

Strains		Phadebact reagent				No Reaction	Multiple Reactions
Group	Number	A	B	C	G		
A	36	28	0	0	0	0	8
B	28	0	28	0	0	0	0
C	24	0	0	21	0	0	3
D	7	1	0	1	1	4	0
E	4	0	0	0	0	4	0
F	5	0	0	0	0	5	0
G	26	0	0	0	22	0	4

Part B Reactions after trypsinization

Group	Number	A	B	C	G	No Reaction	Multiple Reactions
A	8	8	0	0	0	0	0
C	3	0	0	3	0	0	0
G	4	0	0	0	4	0	0

Table 17. Reactions of Supernatants of Overnight Broth Cultures of Beta-Hemolytic Streptococci with Coagglutination Grouping Reagents

Strains		Phadebact Reagent				No	Multiple
Group	Number	A	B	C	G	Reaction	Reactions
A	36	36	0	0	0	0	0
B	28	0	28	0	0	0	0
C	24	1	0	23	0	0	0
D	7	0	0	1	1	5	0
E	4	0	0	0	0	4	0
F	5	0	0	0	0	5	0
G	26	0	0	0	26	0	0

Table 18. Latex Agglutination Reactions Obtained with Extracts of *Beta-Hemolytic Streptococci* (Blood Agar Plate Growth)

Strep.	Number	Pronase Extract		Autoclave Extract	
Group	Tested	No. correct	No. incorrect	No. correct	No. incorrect
A	39	39	0	39	0
B	34	34	0	34	0
C	22	22	0	22	0
F	10	10	0	10	0
G	35	35	0	35	0
None	15	11	4F	14	1F
Total	155	151	4	154	1

Table 19. Latex Agglutination Reaction Obtained with Extracts of Nonbeta-Hemolytic Streptococci (Blood Agar Plate Growth)

Strep. Group	Number Tested	Pronase Extract		Autoclave Extract	
		No. correct	No. incorrect	No. correct	No. incorrect
B	3	3	0	3	0
D faecalis	23	23	0	23	0
D faecium	5	4	0	4	0
D bovis	13	3	10 neg	3	10 neg
C viridans	7	1	6 neg	1	6 neg
F viridans	6	3	3 neg	4	2 neg
G viridans	2	2	0	2	0
None viridans	23	23	0	22	1F
Total	82	63	19	63	19

neg = no reaction with LA reagents

Table 20. Latex Agglutination Reactions Obtained with Extracts of Both Beta- and Nonbeta-hemolytic Streptococci (20 ml THB Growth)

Streptococci		Number Tested	Number Correct	Errors
Beta-hemolytic group:	A	10	10	0
	B	9	9	0
	C	5	5	0
	F	6	6	0
	G	8	8	0
	None	1	1	0
Non beta-hemolytic group:	B	1	1	0
	D	15	13	2 neg[1]
	C	7	1	6 (D)
	F	4	3	1
	None	5	3	2 (D)

[1] neg = no reaction with LA reagents

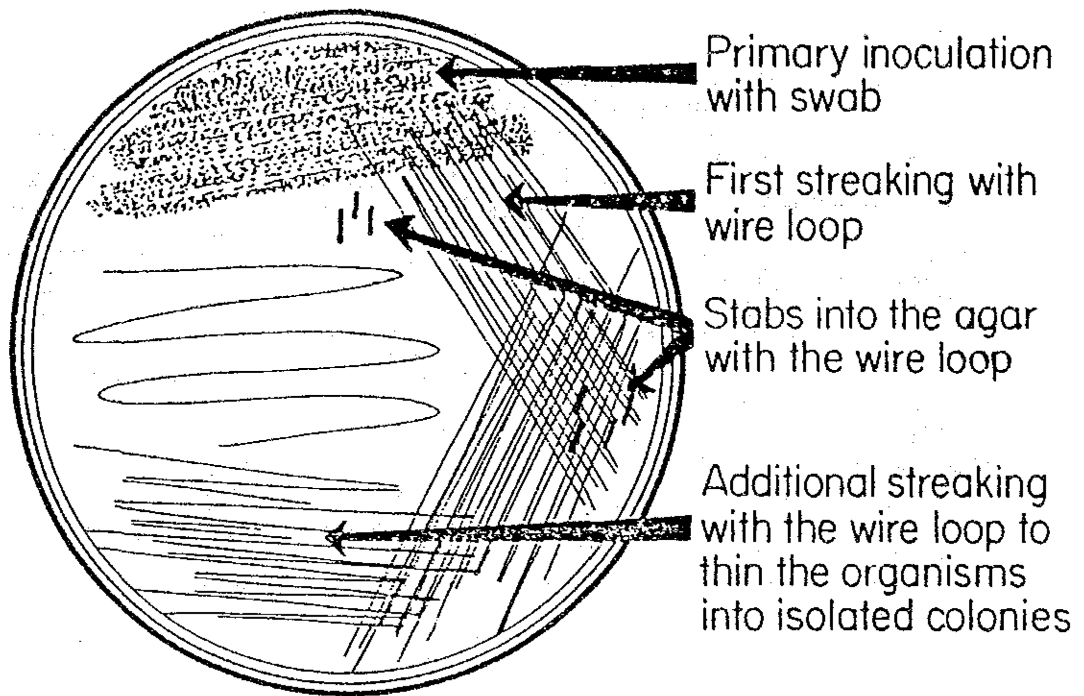


Figure 3. Procedure for preparing a streak-stab culture.

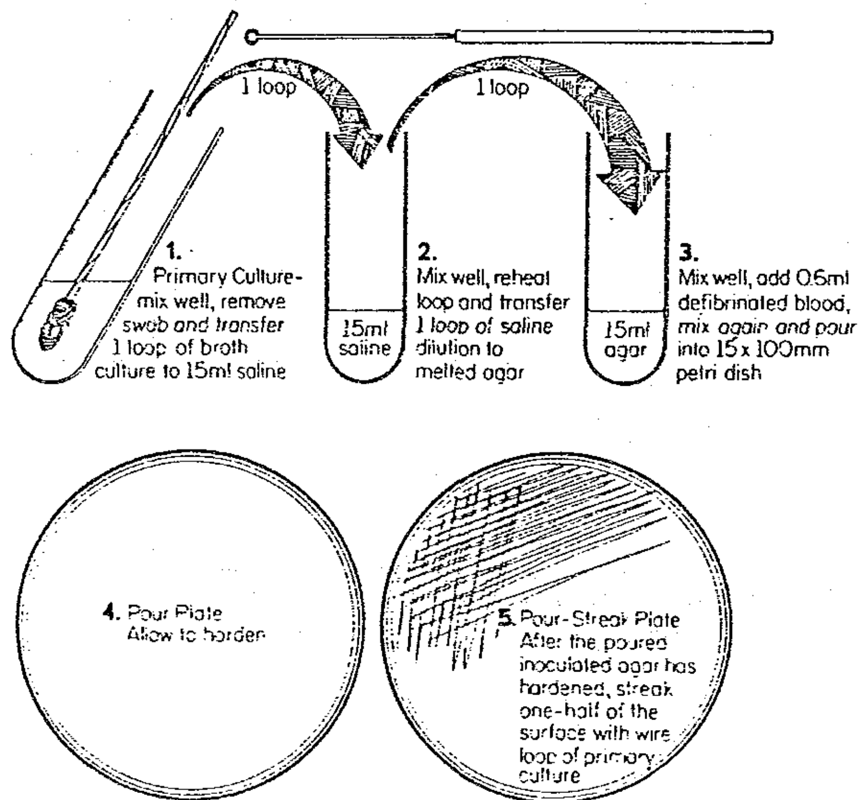


Figure 4. Procedure for preparing pour-plate cultures.

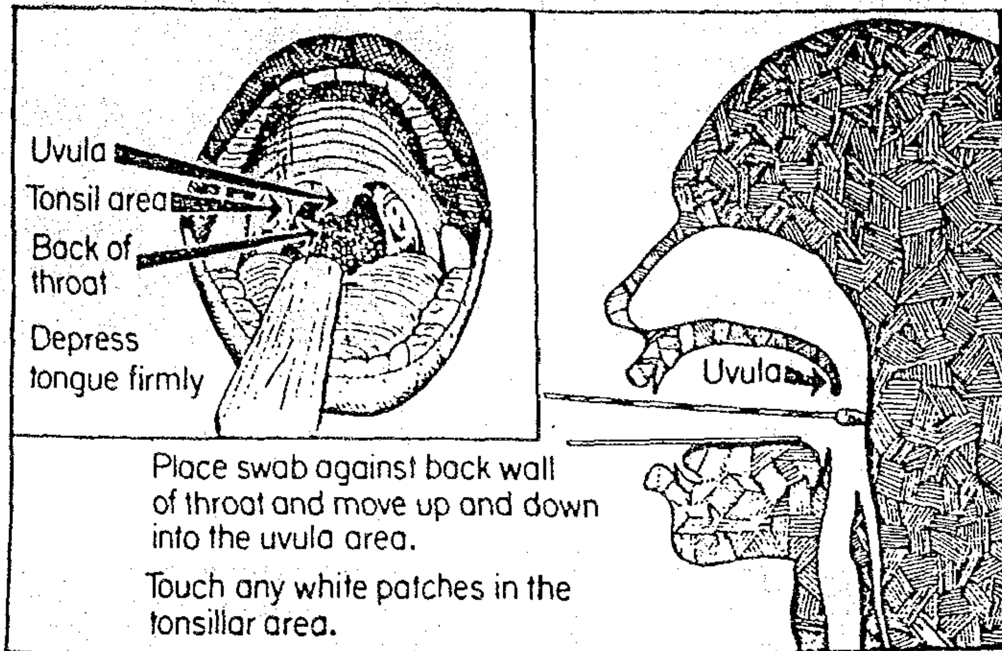


Figure 1. The proper technique for obtaining throat specimens.

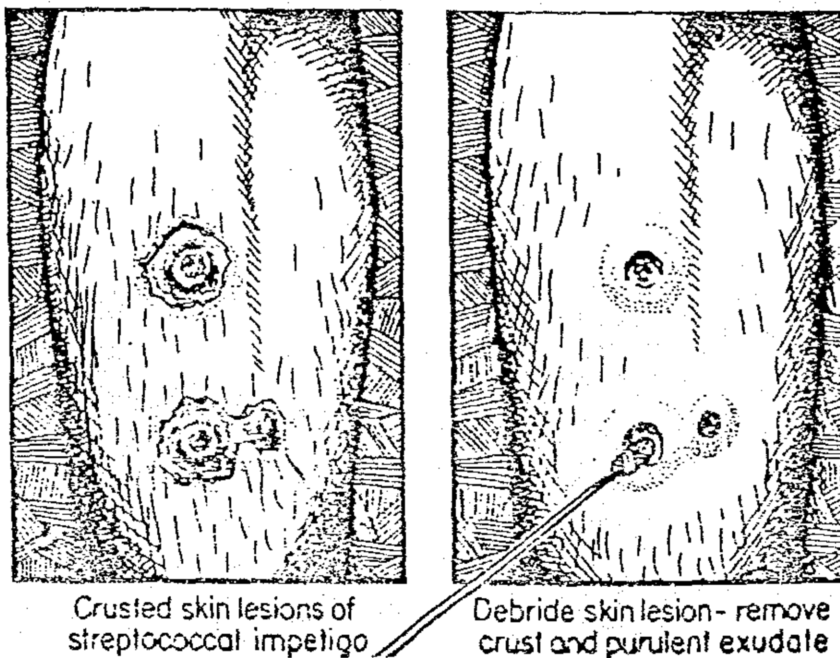
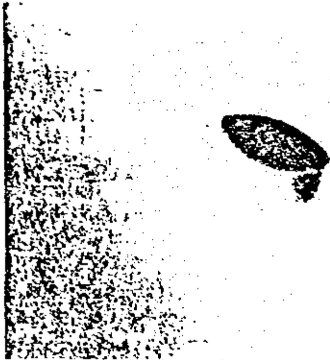


Figure 2. The proper technique for obtaining skin lesion specimens.



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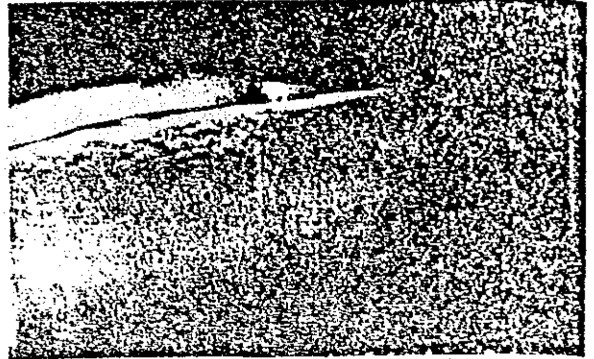

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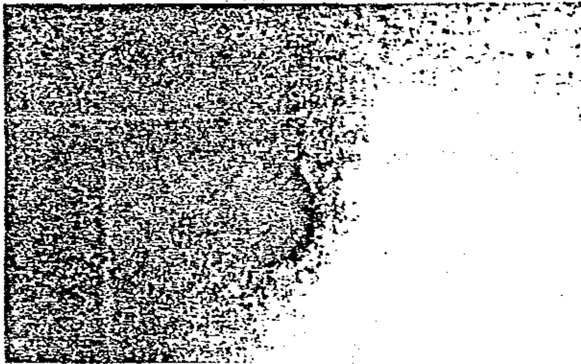

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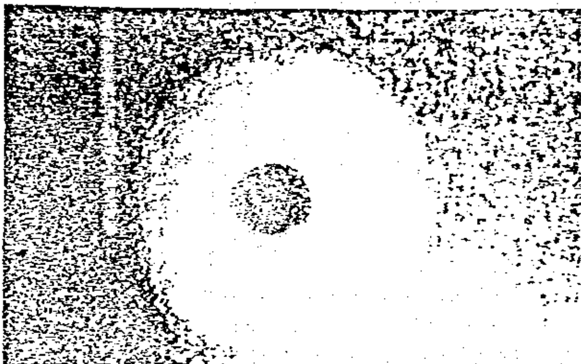

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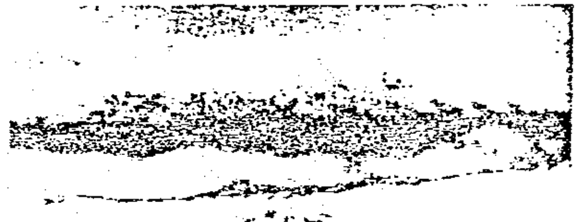
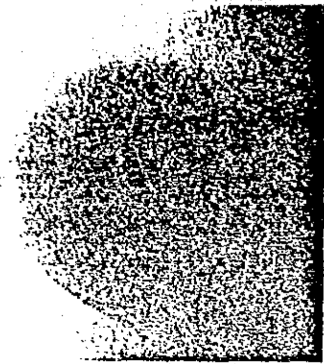


(f)

Figure 5. Streptococcal hemolysis, magnified 35 times.



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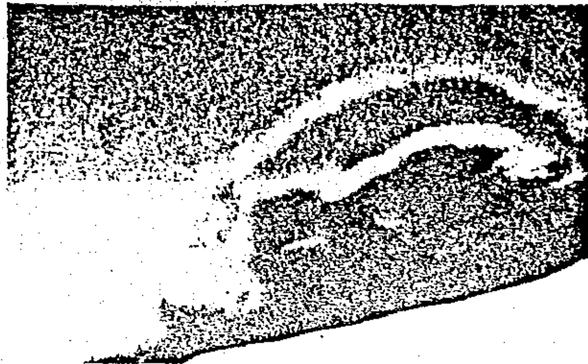

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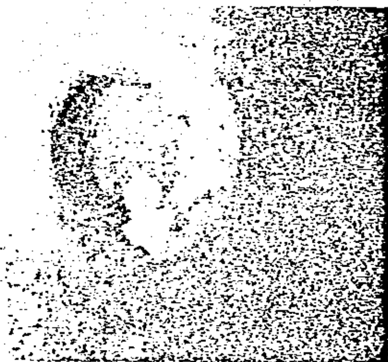


(e)

(f)

Figure 6. Streptococcal hemolysis, magnified 100 times.

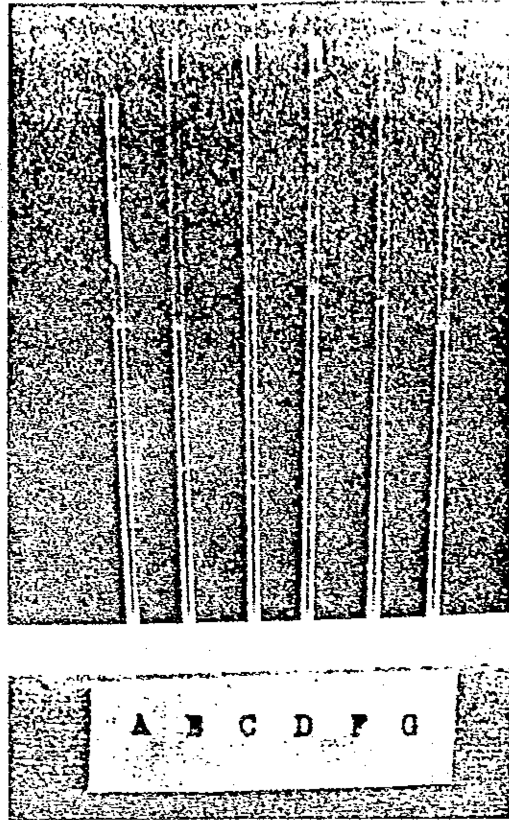


Figure 7. The CDC capillary precipin test.

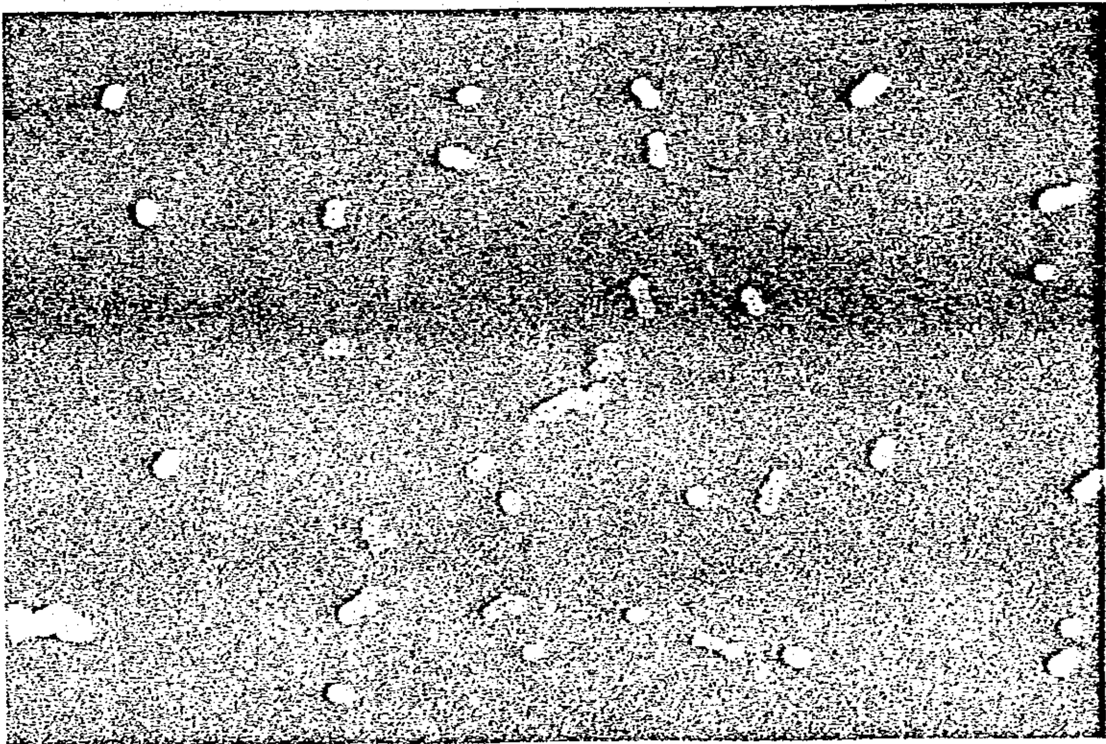
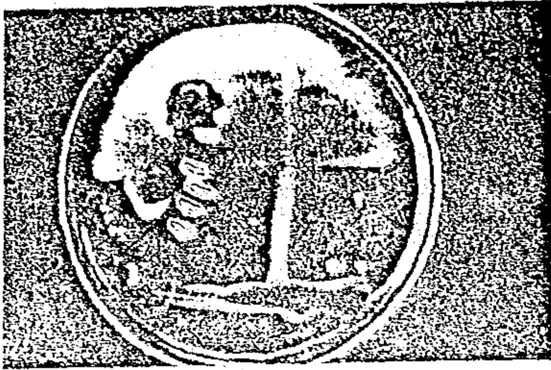


Figure 8. Flourescent antibody reaction of group A streptococci.



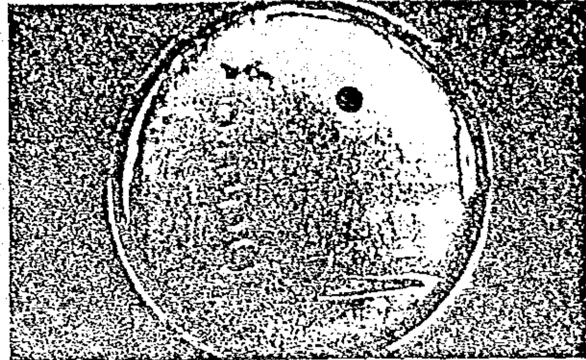


Figure 9. Bacitracin and CAMP tests

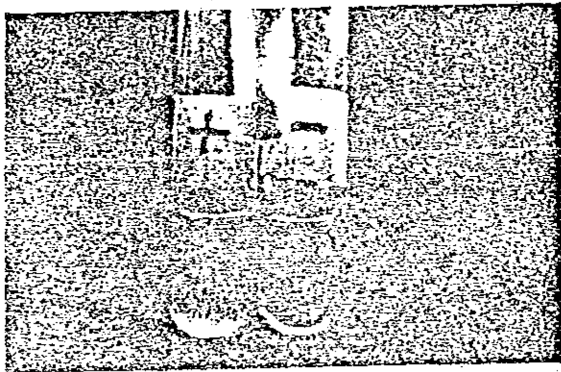


Figure 10. Standard hippurate test

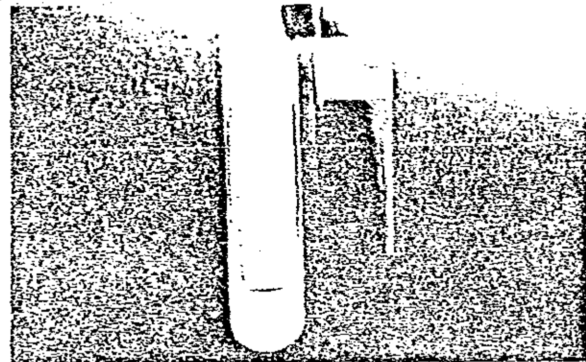


Figure 11. Bile-esculin test

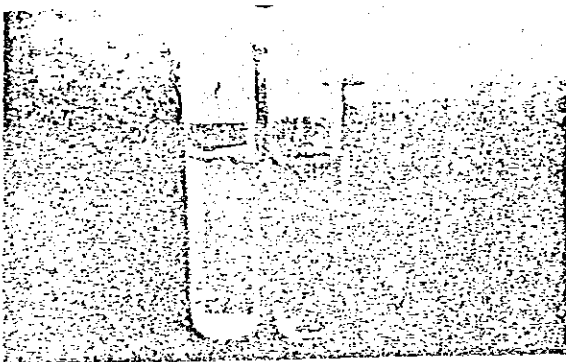


Figure 12. Salt tolerance test

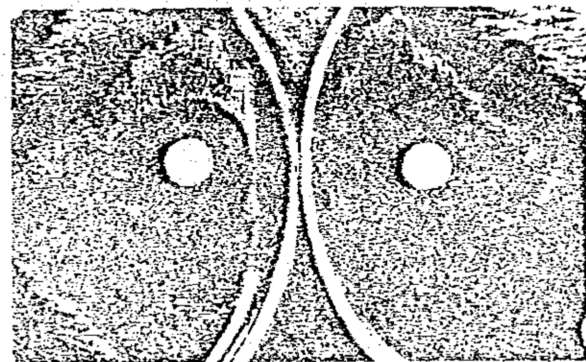


Figure 13. Optochin test for pneumococci

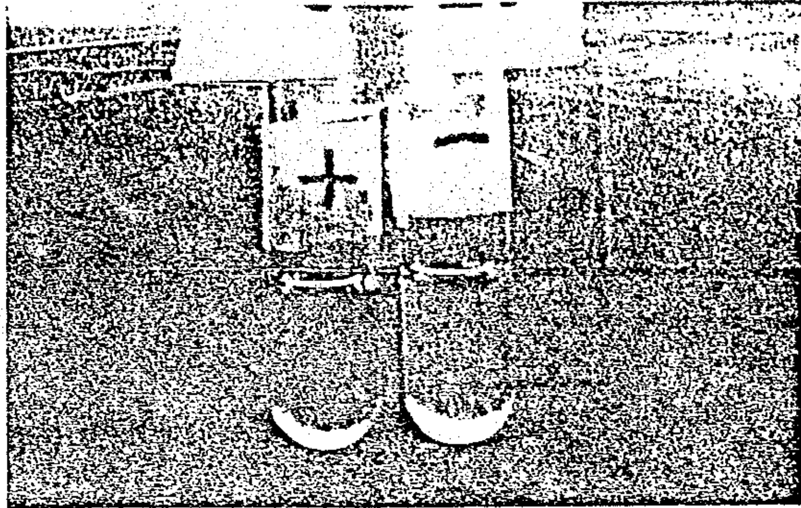


Figure 14. Bile solubility test

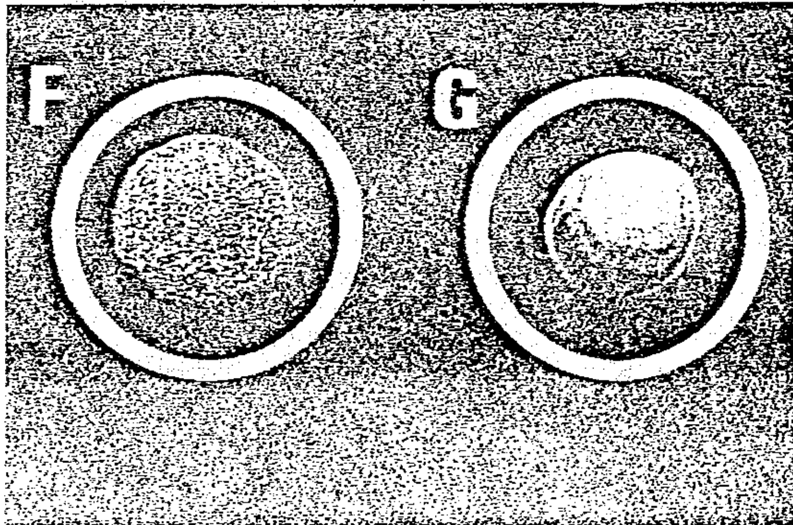


Figure 15. Co- or latex-agglutination reactions of streptococci. Left - positive reaction. Right - negative reaction.

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* Helped to draft the initial document.

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