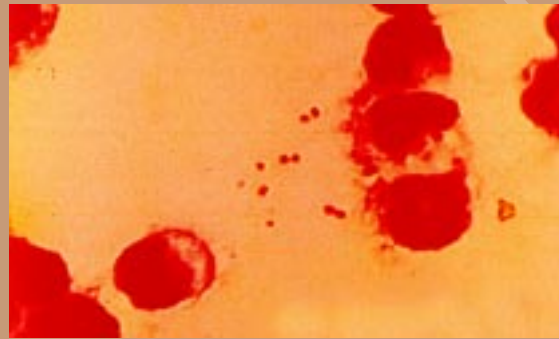
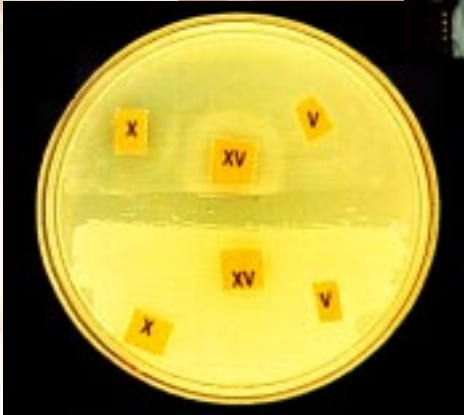


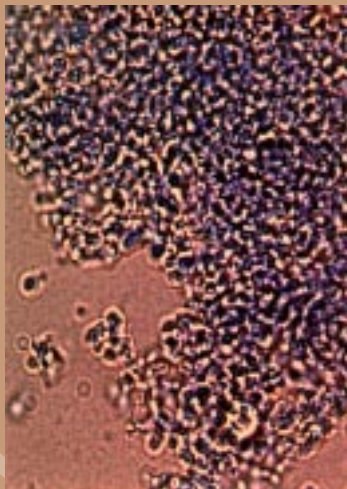
Laboratory Methods for the Diagnosis of

MENINGITIS

Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*,
and *Haemophilus influenzae*



CDC
Centers for Disease Control
and Prevention



August, 1998

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Introduction

This manual summarizes laboratory techniques used in the isolation and identification of *Neisseria meningitidis* (the meningococcus), *Streptococcus pneumoniae* (the pneumococcus) and *Haemophilus influenzae* from the cerebrospinal fluid and blood of patients with clinical meningitis. The procedures described here are not new; most have been used for many years. Even though they require an array of laboratory capabilities, these procedures were selected because of their utility, ease of performance, and ability to give reproducible results. The diversity of laboratory capabilities, the availability of materials and supplies, and their cost, were taken into account. In addition to these basic procedures, methods for subtyping and biotyping of these organisms are included for reference laboratories that have the facilities, the trained personnel, and the desire to perform them.

Acknowledgments

This manual was prepared in collaboration with the following WHO Collaborating Centres: WHO Collaborating Center for Prevention and Control of Epidemic Meningitis, Centers for Disease Control and Prevention, Atlanta, GA, USA; WHO Collaborating Centre for Reference and Research on Meningococci, National Institute of Public Health, Oslo, Norway; and WHO Collaborating Centre for Reference and Research on Meningococci, Institut de Médecine Tropicale du Service de Santé des Armées, Marseilles, France.

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I. Epidemiology of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*

Bacterial meningitis, an infection of the membranes (meninges) and cerebrospinal fluid (CSF) surrounding the brain and spinal cord, is a major cause of death and disability world-wide. Beyond the perinatal period, three organisms, transmitted from person to person through the exchange of respiratory secretions, are responsible for most cases of bacterial meningitis: *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*. The etiology of bacterial meningitis varies by age group and region of the world. Worldwide, without epidemics one million cases of bacterial meningitis are estimated to occur and 200,000 of these die annually. Case-fatality rates vary with age at the time of illness and the species of bacterium causing infection, but typically range from 3 to 19% in developed countries. Higher case-fatality rates (37-60%) have been reported in developing countries. Up to 54% of survivors are left with disability due to bacterial meningitis, including deafness, mental retardation, and neurological sequelae.

Two clinically overlapping syndromes – meningitis and bloodstream infection (meningococcaemia) - are caused by infection with *N. meningitidis* (meningococcal disease). While the two syndromes may occur simultaneously, meningitis alone occurs most frequently. *N. meningitidis* is classified into serogroups based on the immunological reactivity of the capsular polysaccharide. Although 13 serogroups have been identified, the three serogroups A, B and C account for over 90% of meningococcal disease. Meningococcal disease differs from other leading causes of bacterial meningitis because of its potential to cause large-scale epidemics. A region of sub-Saharan Africa extending from Ethiopia in the East to The Gambia in the West and containing fifteen countries and over 260 million people is known as the “meningitis belt” because of its high endemic rate of disease with superimposed, periodic, large epidemics caused by serogroup A, and to a lesser extent, serogroup C. During epidemics, children and young adults are most commonly affected, with attack rates as high as 1,000/100,000 population, or 100 times the rate of sporadic disease. The highest rates of endemic or sporadic disease occur in children less than 2 years of age. In developed countries, endemic disease is generally caused by serogroups B and C. Epidemics in developed countries are typically caused by serogroup C although epidemics due to serogroup B have also occurred in Brazil, Chile, Cuba, Norway and more recently in New Zealand.

Meningitis caused by *H. influenzae* occurs mostly in children under the age of 5 years, and most cases are caused by organisms with the type b polysaccharide capsule (*H. influenzae* type b, Hib). While most children are colonized with a species of *H. influenzae*, only 2-15% harbour Hib. The organism is acquired through the respiratory route. It adheres to the upper respiratory tract epithelial cells and colonizes the nasopharynx. Following acquisition of Hib, illness results when the organism is able to penetrate the respiratory mucosa and enters the blood stream. This is the result of a combination of factors, and subsequently the organism gains access to the CSF, where infection is established and inflammation occurs. An essential virulence factor which plays a major role in determining the invasive potential of an organism is the polysaccharide capsule of Hib. Meningitis is the most severe form of Hib disease; in most countries, however more cases and deaths are due to pneumonia than to meningitis.

Meningitis in individuals at the extremes of age infants, young children and the elderly is commonly caused by *S. pneumoniae*. Younger adults with anatomic or functional asplenia, haemoglobinopathies, such as sickle cell disease, or who are otherwise immunocompromised, also have an increased susceptibility to *S. pneumoniae* infection. *S. pneumoniae*, like Hib, is acquired through the respiratory route. Following the establishment of nasopharyngeal colonization, illness results once bacteria evade the mucosal defences, thus accessing the bloodstream, and eventually reaching the meninges and CSF. As is the case with Hib, many more cases and deaths are due to pneumococcal pneumonia, even though pneumococcal meningitis is the more severe presentation of pneumococcal disease.

The risk of secondary cases of meningococcal disease among close contacts (i.e. household members, day-care centre contacts, or anyone directly exposed to the patient's oral secretions) is high. Antimicrobial chemoprophylaxis with a short course of oral rifampin, a single oral dose of ciprofloxacin, or a single injection of ceftriaxone is effective in eradicating nasopharyngeal carriage of *N. meningitidis*. Although very effective in preventing secondary cases, antimicrobial chemoprophylaxis is not an effective intervention for altering the course of an outbreak. In epidemics, mass chemoprophylaxis is not recommended.

Vaccines have an important role in the control and prevention of bacterial meningitis. Vaccines against *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* are currently available, but the protection afforded by each vaccine is specific to each bacterium and restricted to some of the serogroups or serotypes of each bacterium. For example, vaccines are currently available to prevent *H. influenzae* infections due to serotype b (Hib) but not those infections due to other serotypes or unencapsulated organisms (i.e. nontypeable *H. influenzae*). In addition to establishing a diagnosis, an important role for the laboratory, therefore, is to determine the bacteria and serogroups/serotypes that are causing meningitis in a community.

In industrialized countries, routine use of polysaccharide-protein Hib conjugate vaccines for immunization of infants has almost eliminated Hib meningitis and other forms of severe Hib disease. Several studies in developing countries have corroborated these findings. Pneumococcal polysaccharide vaccines have been used to prevent disease in the elderly and in persons with chronic illnesses that may impair their natural immunity to pneumococcal disease. Meningococcal polysaccharide vaccines are generally used in response to epidemics and for the prevention of disease in overseas travellers although other uses are currently under investigation.

In addition to the existing armamentarium of vaccines, new generation vaccines against meningococcal and pneumococcal disease are under development and evaluation. These vaccines may provide a high degree of protection and broad coverage in all age groups. Until these vaccines become widely available, the current vaccines should be used appropriately and efficiently. Use of any of these vaccines will require laboratory identification of the agents causing disease in addition to epidemiological information about the age and risk groups that are most affected.

II. General Considerations

This manual provides recommendations for the use of media and reagents in the isolation and identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. Alternatively, other media and reagents not listed in this manual may be substituted where appropriate. When making changes outside of these recommendations, it may be helpful for the laboratory staff to consult the authors of this manual.

All of the assays described in this manual should be conducted at Biosafety Level 2. Please refer to the World Health Organization (WHO) Laboratory Biosafety Manual for detailed descriptions of safety precautions.

Antimicrobial susceptibility testing of these organisms is not addressed in this manual, and readers are referred to special textbooks and manuals (i.e. the Manual of Clinical Microbiology, the Clinical Microbiology Procedures Handbook, or the WHO Manual for the National Surveillance of Antimicrobial Resistance of *S. pneumoniae* and *H. influenzae*).

A. Record Keeping

It is recommended that all clinical samples be recorded on a form that provides the following information:

1. Patient's name
2. Patient's age and sex
3. Patient's hospital room number or address
4. Physician's name and address or where the physician can be located
5. Anatomical site of specimen collection
6. Date and time of specimen collection
7. Clinical diagnosis and relevant patient history
8. Antimicrobial agents, if any, that the patient is receiving

In addition, each specimen should have a label firmly attached to the specimen container and bearing the following information:

Patient's name: _____

Hospital: _____

Room: _____

Physician: _____

Specimen type: _____

Date and time of collection: _____

Test requested: _____

A registry at the laboratory should include a bound record book listing each clinical sample received, specifying the date received, the referring hospital or physician, the identification of the organism, results of assays performed, and whether the isolate was

sent to the reference laboratory for further studies. In addition, basic information about the patient should also be noted (age, sex, name, address).

III. Collection and Transport of Clinical Specimens

The collection of clinical specimens is important in the isolation and identification of bacterial agents that cause meningitis. It is recommended that clinical specimens be obtained before antimicrobial therapy is begun to avoid loss of viability of the etiological agents. Treatment of the patient, however, should not be delayed while awaiting collection of specimens. CSF and blood should be processed in a bacteriology laboratory as soon as possible.

A. Collection of Cerebrospinal Fluid (CSF)

The collection of CSF is an invasive technique and should be performed by experienced personnel under aseptic conditions. If meningitis is suspected, CSF is the best clinical specimen to use for isolating and identifying the etiological agents. The collection of CSF should be performed for diagnosis only. CSF should be inoculated directly onto both a supplemented chocolate agar plate (CAP) and a blood agar plate (BAP).

A1. Lumbar Puncture

Usually, 3 tubes of CSF are collected for chemistry, microbiology, and cytology. If only one tube of fluid is available, it should be given to the microbiology laboratory. If more than one tube (1 ml each) is available, the second or third tube should go to the microbiology laboratory.

The kit for collection of CSF (Figure 1) should contain:

1. skin disinfectant
2. sterile gauze and Band-Aid
3. lumbar puncture needles: 22 gauge/3.5" for adults; 23 gauge/2.5" for children
4. sterile screw-cap tubes
5. syringe and needle
6. transport container
7. Trans-Isolate (T-I) medium (if CSF cannot be analysed in the microbiological laboratory immediately)

The patient should be kept motionless, either sitting up or laying on the side, with his or her back arched forward so that the head almost touches the knees during the procedure (Figure 2). Disinfect the skin along a line drawn between the crests of the two ilia with 70 % alcohol to clean the surface and remove debris and oils. Then apply tincture of iodine or povidone-iodine. Let dry. The needle is introduced, and the drops of fluid (1 ml minimum, 3-4 ml if possible) are collected into sterile, screw-cap tubes. Label the specimen as described earlier. Do not refrigerate the specimen. Hand carry it (whenever feasible) to the laboratory as soon as possible. Avoid exposure to excessive heat or sunlight.



Figure 1. Kit for collection of CSF.

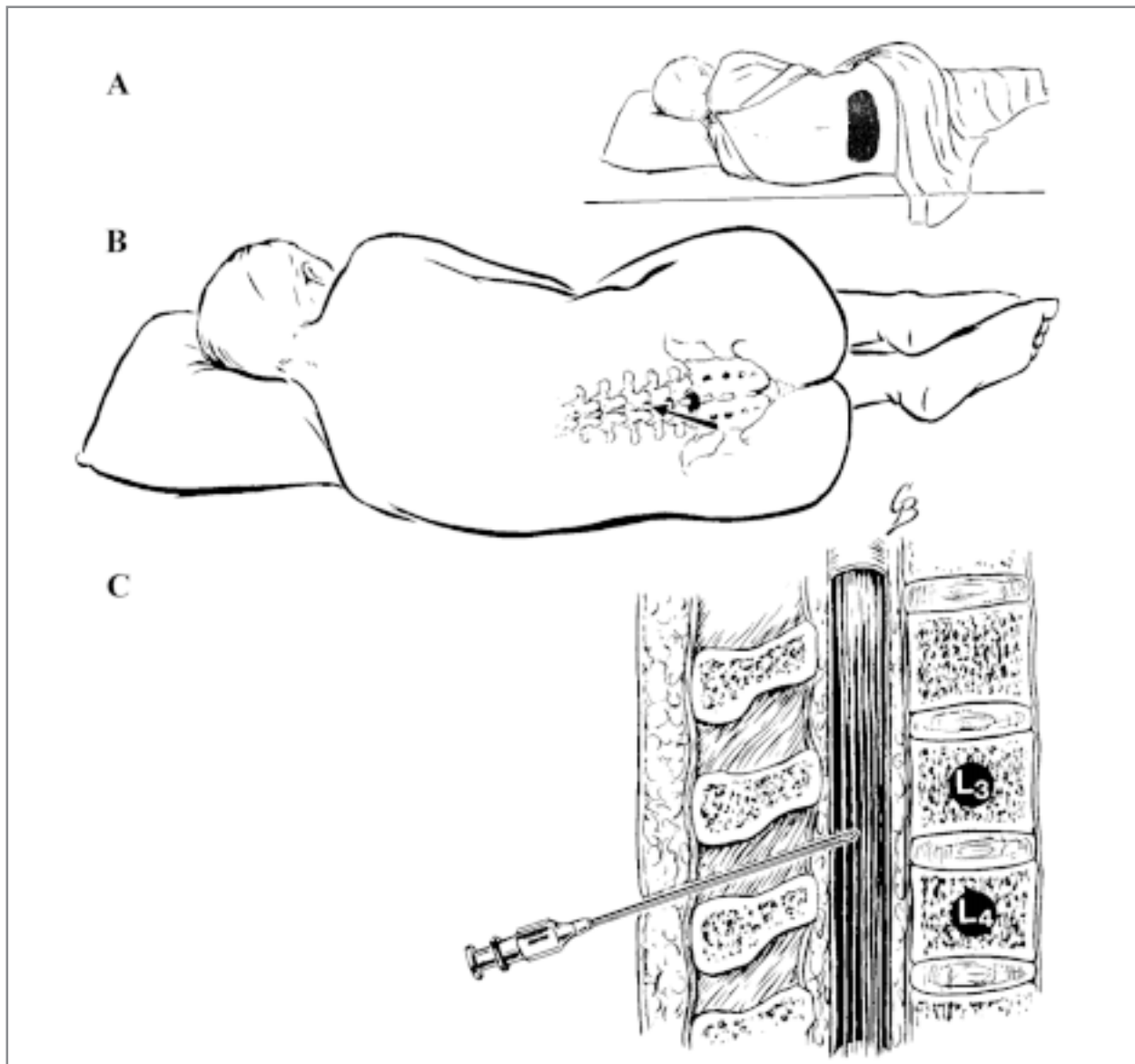


Figure 2. Collection of CSF by lumbar puncture.

- A. The patient lies on his side with knees flexed and back arched to separate the lumbar vertebrae. The patient is surgically draped, and an area overlying the lumbar spine is disinfected.
- B. The space between lumbar vertebrae L3 and L4 is palpated with the sterilely gloved forefinger.
- C. The spinal needle is carefully directed between the spinous processes, through the interspinous processes, through the interspinous ligaments into the spinal canal.

Illustration and procedure reprinted with permission of J. B. Lippincott Company from Koneman, Elmer w. et al. (1992). Diagnostic Microbiology, Page 90.

B. Collection of Blood

For the diagnosis of bacterial meningitis, blood should be collected when a spinal tap is contraindicated or cannot be performed for technical reasons.

B1. Precautions

Infection may be transmitted from patient to staff and from staff to patient during the blood-taking procedure. Viral agents are the greatest hazard and in some instances are potentially lethal. Of particular importance are the viruses causing hepatitis and acquired immunodeficiency syndrome. To decrease the risk of transmission of these viral agents, the recommendations below should be followed:

- (a) Wear latex or vinyl gloves impermeable to liquids.
- (b) Change gloves between patients.
- (c) Inoculate blood into blood culture media immediately to prevent the blood from clotting in the syringe. Syringes and needles should be disposed of in a puncture-resistant, autoclavable container. No attempt should be made to re-cap the needle. A new syringe and needle **must** be used for each patient.
- (d) Wipe the surface of the blood culture bottle and the gloves with a disinfectant.
- (e) Label the bottle.
- (f) For the transport to the microbiology laboratory, place the blood culture medium in a container that can be securely sealed.
- (g) Specimen containers should be individually and conspicuously labelled. Any containers with blood on the outside should be wiped thoroughly. Such containers should be transported in individual plastic envelopes.
- (h) Remove gloves and discard in an autoclavable container.
- (i) Wash hands with soap and water immediately after removing gloves.
- (j) Transport the specimen to the microbiology laboratory or, if that facility is closed, store the specimen in an approved location.
- (k) In the event of a needle-stick injury or other skin puncture or wound, wash the wound liberally with soap and water. Encourage bleeding.
- (l) Report any contamination of the hands or body with blood, or any puncture wound or cut to the supervisor and the health service for treatment.

B2. Sensitivity of Blood Cultures

Several variables affect the sensitivity of blood cultures: the number of collections, the volume of each collection, and the steps taken to inhibit or neutralize bactericidal properties of blood may vary with the age of the patient. It may be difficult to collect a large amount of blood from a child; 1-3 ml is usually sufficient. Blood cultures from young children should be diluted to 1-2 ml of blood in 20 ml of broth (1:10 to 1:20). Blood cultures from adults should be diluted to 5-10 ml of blood in 50 ml of broth (1:5 to 1:10).

Blood should be cultured in trypticase soy broth (TSB) or brain heart infusion with a growth supplement (such as IsoVitaleX or Vitox) to support growth of organisms such as *H. influenzae*. Neutralization of normal bactericidal properties of blood and potential antimicrobial agents is accomplished by adding chemical inhibitors such as 0.025% sodium polyanetholesulfonate (SPS) to culture media and by diluting the blood. SPS,

which has anticoagulant, antiphagocytic, anticomplementary, and antilysozymal activity, may be inhibitory if used in higher concentrations.

B3. Venipuncture

An outline of the proper method for collecting blood from the arm is shown in Figure 3.

- (a) Gather everything needed to complete the process: gloves, syringe, needle, tourniquet, gauze squares, cotton balls, Band-Aid, puncture resistant container, culture medium and antiseptic; iodine tincture or povidone-iodine is preferred, but 70% alcohol is an acceptable alternative. However, alcohol with concentrations greater than 70% should not be used because the increased concentrations result in decreased antibactericidal activity. The size of the needle will depend on the collection site and the size of the vein. A 23-gauge needle that is 20-25 mm in length or a butterfly needle is generally used for children.
- (b) Select an arm and apply a tourniquet to restrict the flow of venous blood. The large veins of the forearm are illustrated in Figure 3. The most prominent vein is usually chosen.
- (c) Vigorously wipe the skin with the 70% alcohol, and swab with the iodine tincture or povidone-iodine. Rub over the selected area. Allow to dry. **If the vein is palpated again, repeat the skin disinfection.**
- (d) After the disinfectant has dried, insert the needle into the vein with the bevel of the needle face up. Once the vein is entered, withdraw the blood by pulling back the barrel of the syringe in a slow, steady manner. Air must **not** be pumped into a vein. After the desired amount of blood is obtained, release the tourniquet and place a sterile cotton ball over the insertion site while holding the needle in place. Withdraw the needle and have patient hold the cotton-ball firmly in place until the wound has stopped bleeding. Inoculate the culture medium. Put the Band-Aid on the wound.
- (e) Vacutainer tubes should be used for blood collection, if available.

C. Transport of Clinical Specimens

S. pneumoniae, *H. influenzae* and *N. meningitidis* are fastidious and fragile bacteria. They are more reliably isolated if the clinical material is examined as soon as possible after collection.

C1. CSF

As soon as the CSF has been collected, it should be transported to the microbiology laboratory, where it should be examined as soon as possible (within one hour from the time of collection) (Figure 4). Do not expose the CSF to sunlight or extreme heat or cold. If *N. meningitidis* is suspected to be the cause of the illness, and a delay of several hours in processing specimens is anticipated, incubating the CSF (with screw-caps loosened) at 35 °C in a 5% CO₂ atmosphere (or candle-jar) may improve bacterial survival. If same-day transport to the laboratory is not possible, CSF should be inoculated aseptically into a Trans-Isolate (T-I) medium with a syringe and held overnight at, or close to, 35 °C. T-I is a biphasic medium that is useful for the primary culture of meningococci and other

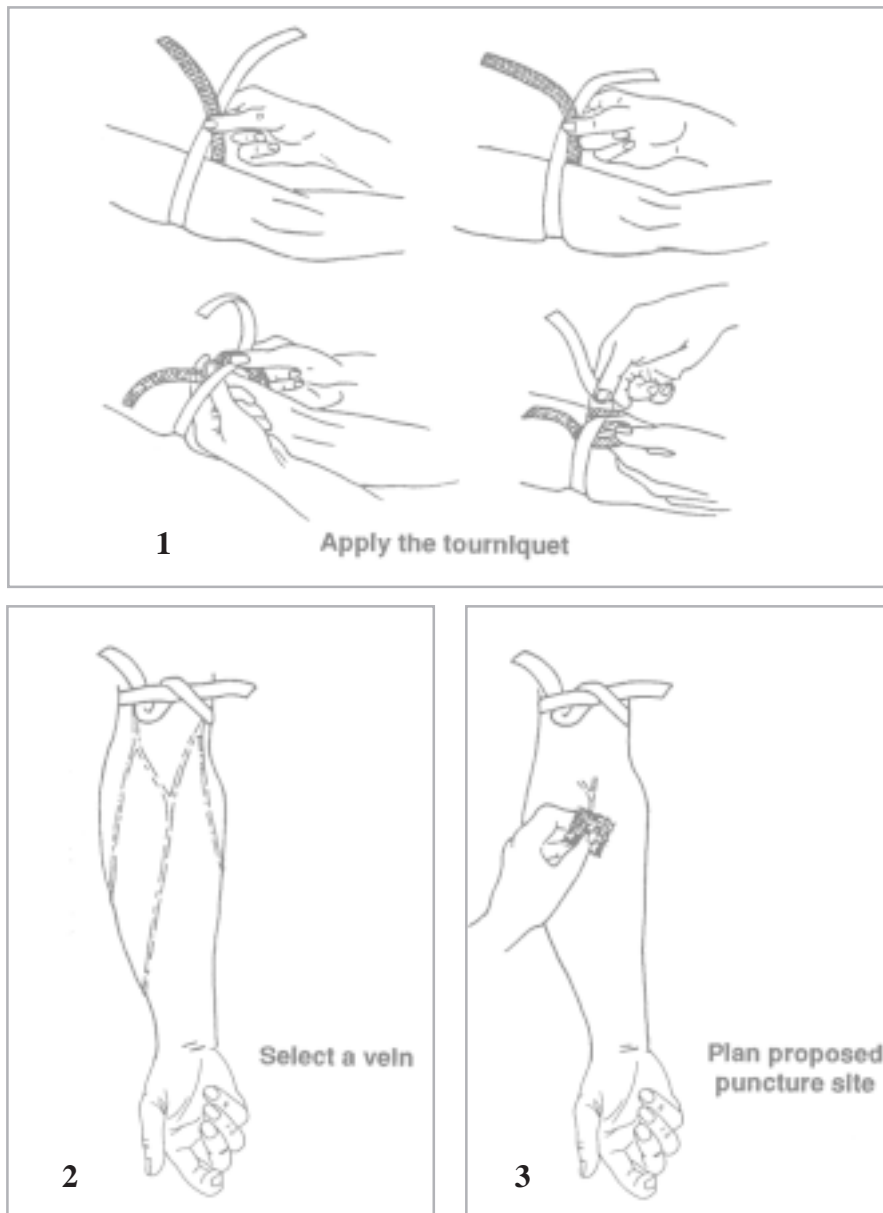


Figure 3. Collection of blood from an arm.

1. Apply the tourniquet
2. Select a vein
3. Plan proposed puncture site

etiological agents of bacterial meningitis from CSF and blood samples (Figure 5). It can be used as a growth medium as well as a holding and transport medium. The preparation of the T-I medium is described in Annex C (C4).

- (a) The T-I bottle septum should be disinfected with alcohol and iodine and allowed to dry before inoculation. Inoculate 1 ml of CSF into the T-I medium, which has either been pre-warmed in the incubator (35 °C-37 °C) or kept at room temperature (25 °C). Keep the remaining CSF in the container or syringe in which it was collected. Do not refrigerate, but hold at room temperature before Gram staining.
- (b) After inoculation, T-I bottles are incubated at 35 °C overnight. Label the T-I bottle appropriately with the patient identification, and date and time of CSF inoculation. Incubate the T-I medium at 35 °C for up to 7 days. Venting the bottle with a venting needle, or a sterile cotton-plugged hypodermic needle after the initial 24-h incubation, or as soon as possible after transportation has been completed, encourages growth and survival. If transport is delayed, vented bottles can be held for days at moderate to warm room temperatures (25 °C-30 °C). The vents must be removed before shipment. It is essential to obtain specimens aseptically and to avoid contamination when inoculating or sampling the bottles.

C2. Blood

- (a) Blood cannot be transported before being placed in broth because the collection procedure does not use an anticoagulant. Blood should be injected into the broth culture medium within one minute of collection. If the blood culture bottle contains a diaphragm, clean the diaphragm with 70% alcohol and povidone-iodine before inoculating the medium. Swirl the bottle several times. Discard the needle and syringe in a puncture-resistant container. Do not re-cap the needle. Clean the diaphragm of the blood culture bottle, if necessary. Then label it appropriately with patient identification and the date and time of blood collection. The preparation of blood culture media is described in Annex C (C2).
- (b) The inoculated medium can be held at room temperature (20 °C-25 °C) for 4 to 6 hours before incubation at 35 °C. Inoculated or uninoculated blood-culture medium must **not** be placed in a refrigerator. A portable incubator may be used (temperature range 25 °C-35 °C).
- (c) Immediately transport the inoculated media to the laboratory. All inoculated blood-culture media should be received by the laboratory within 12 to 18 hours for subculture and should be protected from temperature extremes (less than 18 °C, more than 37 °C) with a transport carrier such as Styrofoam, which can keep the samples at moderate temperature.

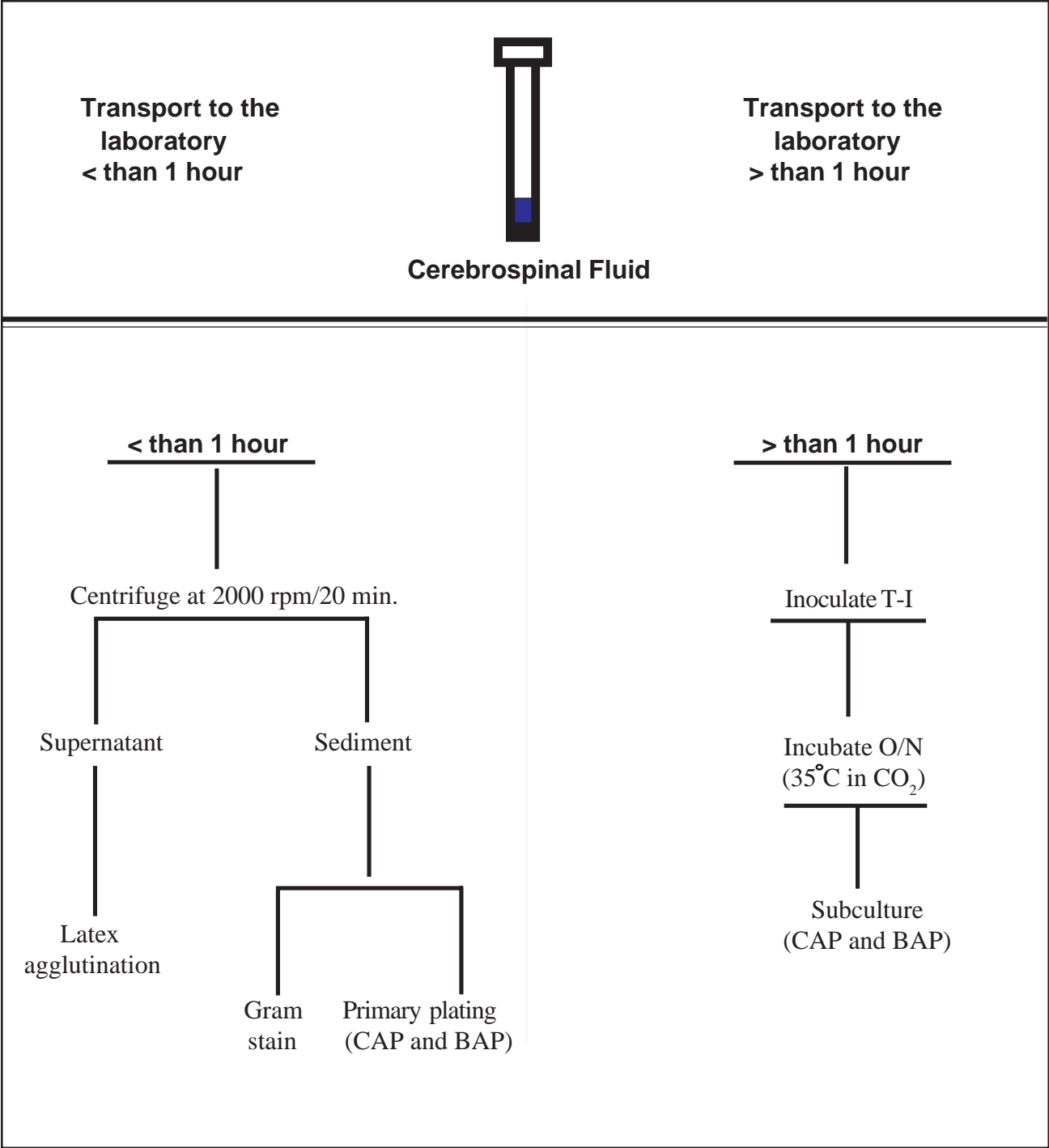


Figure 4. Processing of CSF.



Figure 5. Trans-Isolate medium.

IV. Primary Culture, Subculture, and Presumptive Identification

A. Inoculation of Primary Culture Media

A1. CSF

Once the CSF has arrived at the microbiology laboratory, centrifuge it for 20 minutes at 2000 rpm. Draw off the supernatant with a Pasteur pipette. When antigen detection by latex agglutination is planned, save the supernatant. Sediment must be either vigorously vortexed or well mixed. Use one or two drops of sediment to prepare the Gram stain and use 1 drop to streak the primary culture media (Figure 4).

Note: If less than 1 ml of CSF is available, do not centrifuge. Use it for the Gram stain and plate it directly.

The best medium for *S. pneumoniae* is a BAP, which is a trypticase soy agar (TSA) plate containing sheep or horse blood (5%). Human blood is not an acceptable substitute. For *H. influenzae*, a CAP supplemented with haemin and a growth supplement such as IsoVitaleX, supplement B, or Vitox should be used. An acceptable alternative to achieve growth of *H. influenzae* on BAP is cross-streaking the medium with *Staphylococcus aureus* or applying a filter paper/disk saturated with X and V factors to the surface of the medium, after the medium had been inoculated. *H. influenzae* forms satellite colonies along the length of the staphylococcal growth or produces a halo of growth around the XV strip/disk. *N. meningitidis* grows on both BAP and CAP, as will *S. pneumoniae*. If only one type of plate is available, choose CAP, because all three etiological agents can grow on it. A bacteriological loop is used to streak or thin the bacteria into single colonies. BAP that have been properly streaked are shown in Figures 6 and 7, and properly streaked CAP is shown in Figure 8. The agar plates should be incubated in a 5% CO₂ incubator or candle-jar. A backup broth (e.g. brain heart infusion broth) should be inoculated with some of the pellet and also incubated.

When T-I medium was used for transport, after 24 hours of incubation, with a sterile needle and syringe, transfer 100 µl of the liquid portion of T-I onto BAP and CAP, and streak for isolation.

1.1. Gram Stain Procedure for CSF (Hucker Modification)

A presumptive diagnosis of bacterial meningitis caused by *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* can be made by Gram stain of the CSF sediment or by detection of specific antigens in the CSF by a latex agglutination test. Positive results of either or both tests can provide evidence of infection, even if cultures fail to grow.

- (a) Centrifuge the CSF for 20 minutes at 2000 rpm.
- (b) Prepare smear by placing 1 or 2 drops of sediment on an alcohol-rinsed and dried slide, allowing drop(s) to form one large drop. Do not spread fluid nor use too heavy a concentration of sediment.
- (c) Air dry the slide in a biosafety cabinet, if available.
- (d) Pass the slide quickly through a flame three times to fix the smear. Do not flame until dry. Alternatively, fixation by methanol (95%-100%) can be used.
- (e) Flood the smear with ammonium oxalate-crystal violet and let stand for 1 minute.

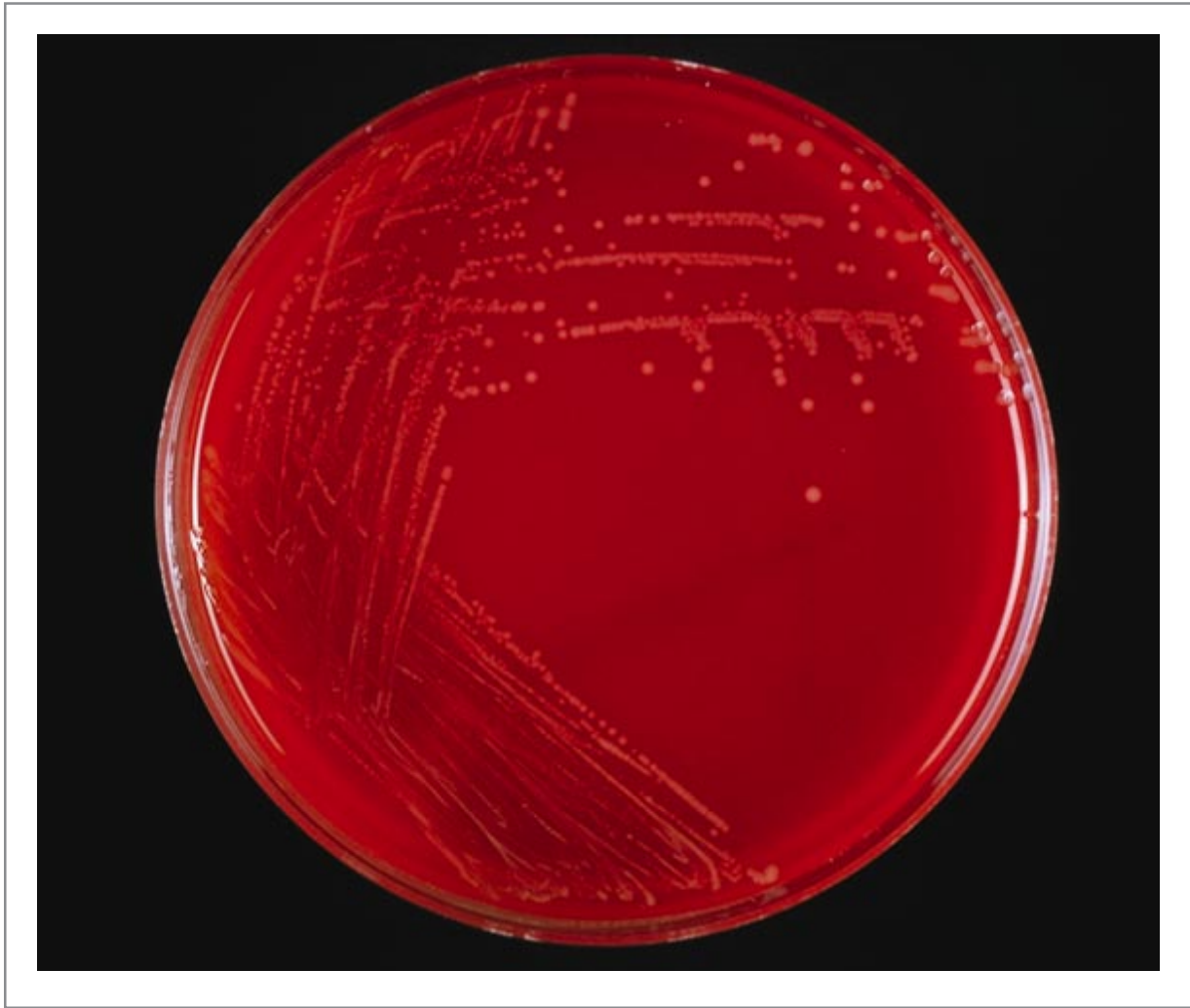


Figure 6. *N. meningitidis* - proper streaking and growth on blood agar plate.

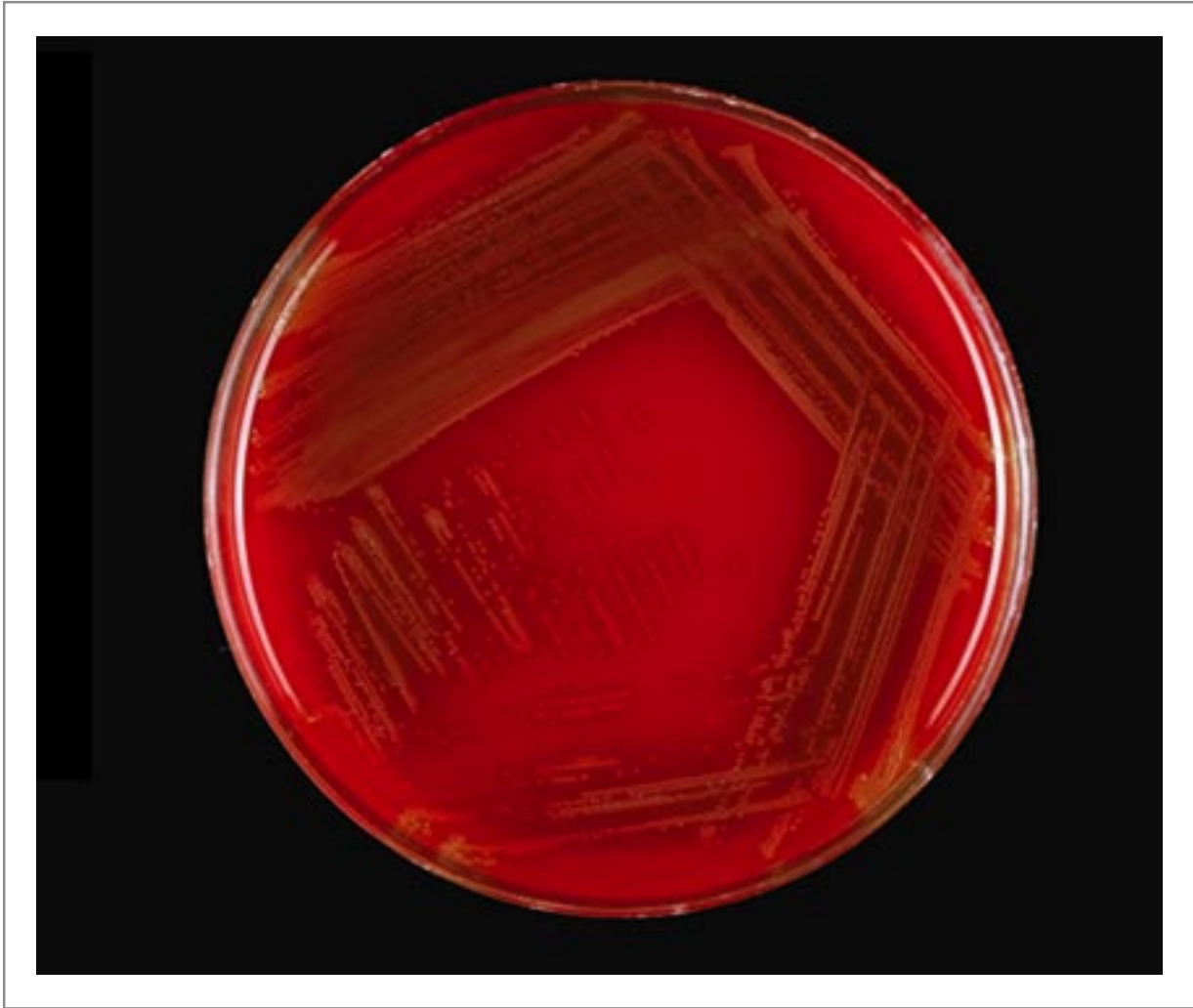


Figure 7. *S. pneumoniae* - proper streaking and growth on blood agar plate.

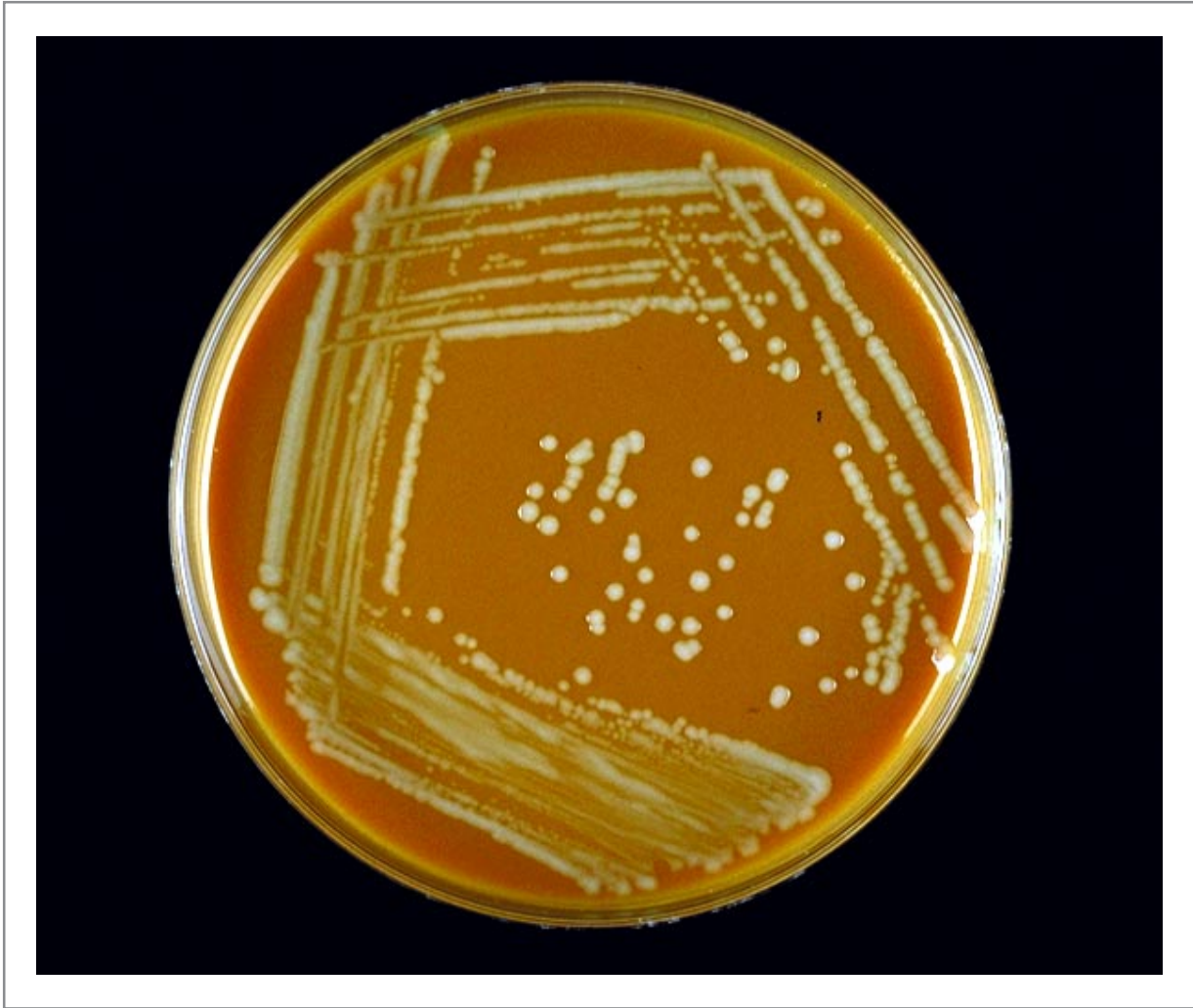


Figure 8. *H. influenzae* - proper streaking and growth on chocolate agar plate.

- (f) Rinse gently with tap water. Drain off excess water.
- (g) Flood the smear with Gram's iodine solution and let stand for 1 minute.
- (h) Rinse gently with tap water and drain.
- (i) Decolorize with 95% ethyl alcohol (5-10 seconds may be enough).
- (j) Counterstain with safranin for 20-30 seconds, or with carbol-fuchsin for 10-15 seconds.
- (k) Rinse the slide with tap water and blot dry.
- (l) Examine the stained smear microscopically, using a bright-field condenser and an oil-immersion lens.

N. meningitidis may occur intra- or extra-cellularly in the polymorphonuclear leukocytes and will appear as Gram-negative, coffee-bean-shaped diplococci (Figure 9a). *S. pneumoniae* are lanceolate, Gram-positive diplococci sometimes occurring in short chains (Figure 9b). *H. influenzae* are small, pleomorphic Gram-negative rods or coccobacilli with random arrangements (Figure 9c). Other manuals should be consulted for Gram stain reactions of other bacteria.

1.2. General Method for Performing Latex Agglutination Tests

Several commercial kits are available. **Follow the manufacturer's instructions precisely when using these tests.** General recommendations and instructions typical for the detection of soluble bacterial antigens are provided here. For best results, test the supernatant of the centrifuged CSF sample as soon as possible. If immediate testing is not possible, the sample can be refrigerated (between 2 •C and 8 •C) up to several hours, or frozen at -20 •C for longer periods. Reagents should be kept refrigerated between 2 •C and 8 •C when not in use. Product deterioration occurs at higher temperatures, especially in tropical climates, and test results may become unreliable before the expiration date of the kit. Latex suspensions should never be frozen.

Performance of the Test

- (a) Heat the supernatant of the CSF in a boiling water bath for 5 minutes.
- (b) Shake the latex suspension gently until homogenous.
- (c) Place one drop of each latex suspension on a ringed glass slide or a disposable card.
- (d) Add 30-50 µl of the CSF to each suspension.
- (e) Rotate by hand for 2-10 minutes. Mechanical rotation at 100 rpm, if available, is recommended.

Reading the Test Results

Read under a bright light without magnification.

Negative reaction: the suspension remains homogenous and slightly milky in appearance.

Positive reaction: agglutination (or visible clumping) of the latex particles occurs within 2 minutes,.

Note: Counter immunoelectrophoresis may also be used for direct antigen detection in CSF.

A2. Blood

The blood culture bottles are inoculated directly with blood and should be vented before incubation at 35 •C-37 •C. This is accomplished by inserting a sterile cotton-plugged needle into the rubber part of the blood culture bottle.

B. Subculture

Since the primary purpose of this manual is to aid in the identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*, the methods described here will not allow for identifying other isolates that may be of clinical importance but more rarely encountered. Microbiologists should refer to clinical microbiology manuals, such as the American Society for Microbiology's Manual of Clinical Microbiology, for procedures to identify other bacteria.

B1. Blood Culture Bottle

Examine the blood culture bottles at 14 to 17 hours and then every day for up to 7 days. Any turbidity or lysis of the erythrocytes may be indicative of growth, and subcultures should be made immediately. Since these three organisms are fragile, subcultures should be performed after 14 to 17 hours of incubation, again at 48 hours, and at day 7, **regardless of the appearance of the blood culture bottles**, since the absence of turbidity does not always correlate with the absence of bacterial growth. Before subculturing, swirl the bottle to mix the contents.

Subcultures are made by first disinfecting the surface of the blood culture bottle rubber stopper with alcohol and a povidone-iodine swab, and then aspirating a small volume (0.5 ml) with a syringe and needle from the blood culture bottle and inoculating the agar media with the fluid. Ordinarily, both CAP and BAP are used for subculture. When only one agar plate is used, it should be CAP, since CAP contains growth factors needed for *H. influenzae* whereas BAP does not. The agar plates should be streaked as shown in Figures 6, 7 and 8, and incubated for up to 48 hours. When bacterial growth has been confirmed by subculture of the blood culture bottle, there is no need to continue to incubate the bottle. The bottle should be disposed of according to safety procedures.

B2. T-I Medium

- (a) After 24 hours of incubation, using a sterile needle and syringe, transfer 100 µl of the liquid portion of T-I onto BAP and CAP, streak the plate, and incubate at 35 • C in a CO₂ atmosphere for up to 48 hours.
- (b) Check for purity of the growth by performing Gram stain.
- (c) If no growth occurs, subculture the T-I medium at 3 days and again at 7 days.

C. Macroscopic Examination of Colonies

N. meningitidis grows on BAP, but *H. influenzae* does not. When grown on CAP, *H. influenzae* and *N. meningitidis* look similar, but the two can be distinguished: *H. influenzae* gives off a pungent smell of indol, while *N. meningitidis* does not.

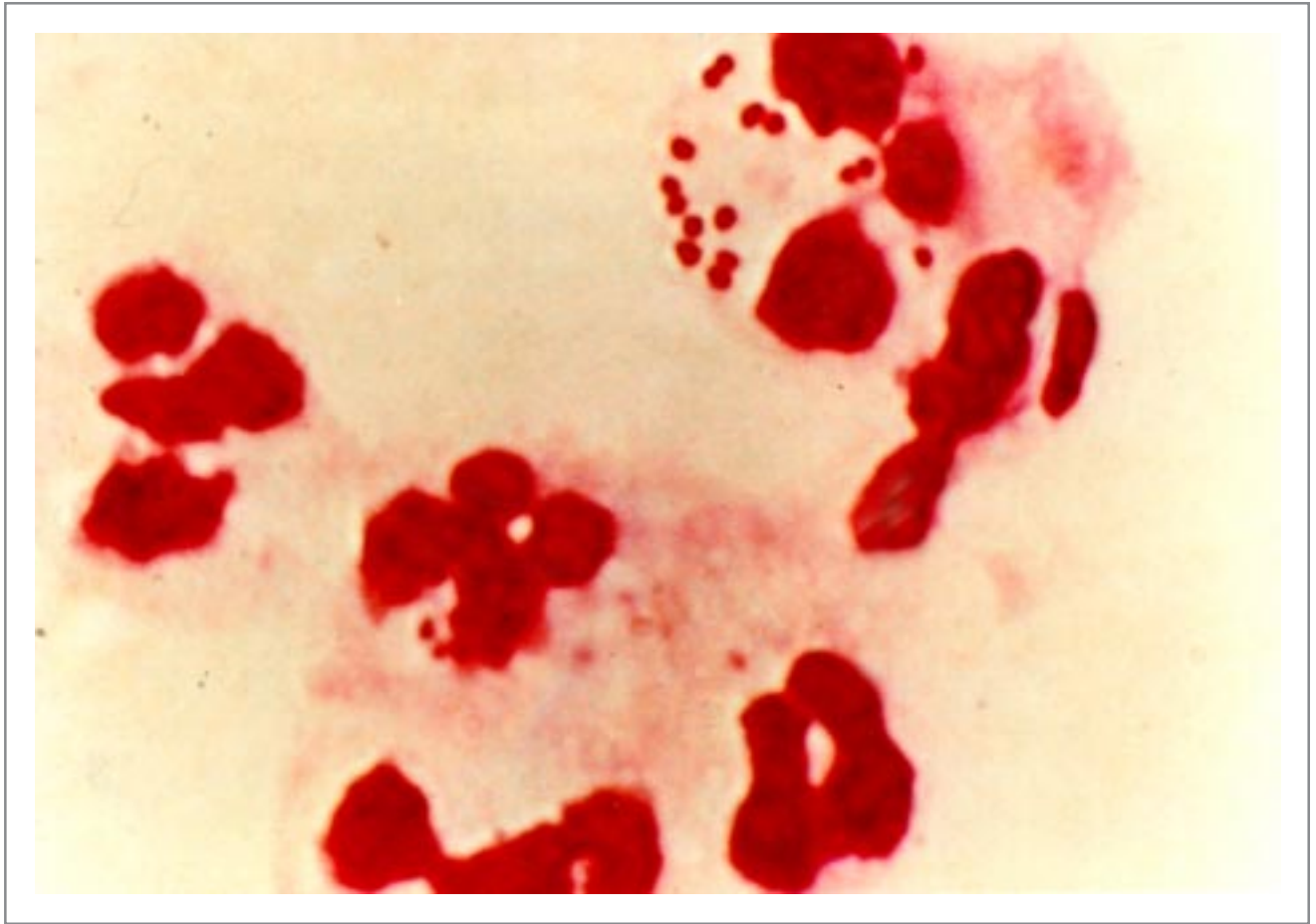


Figure 9a. Gram stain of cerebrospinal fluid: *N. meningitidis*: intra-cellular, gram-negative diplococci.

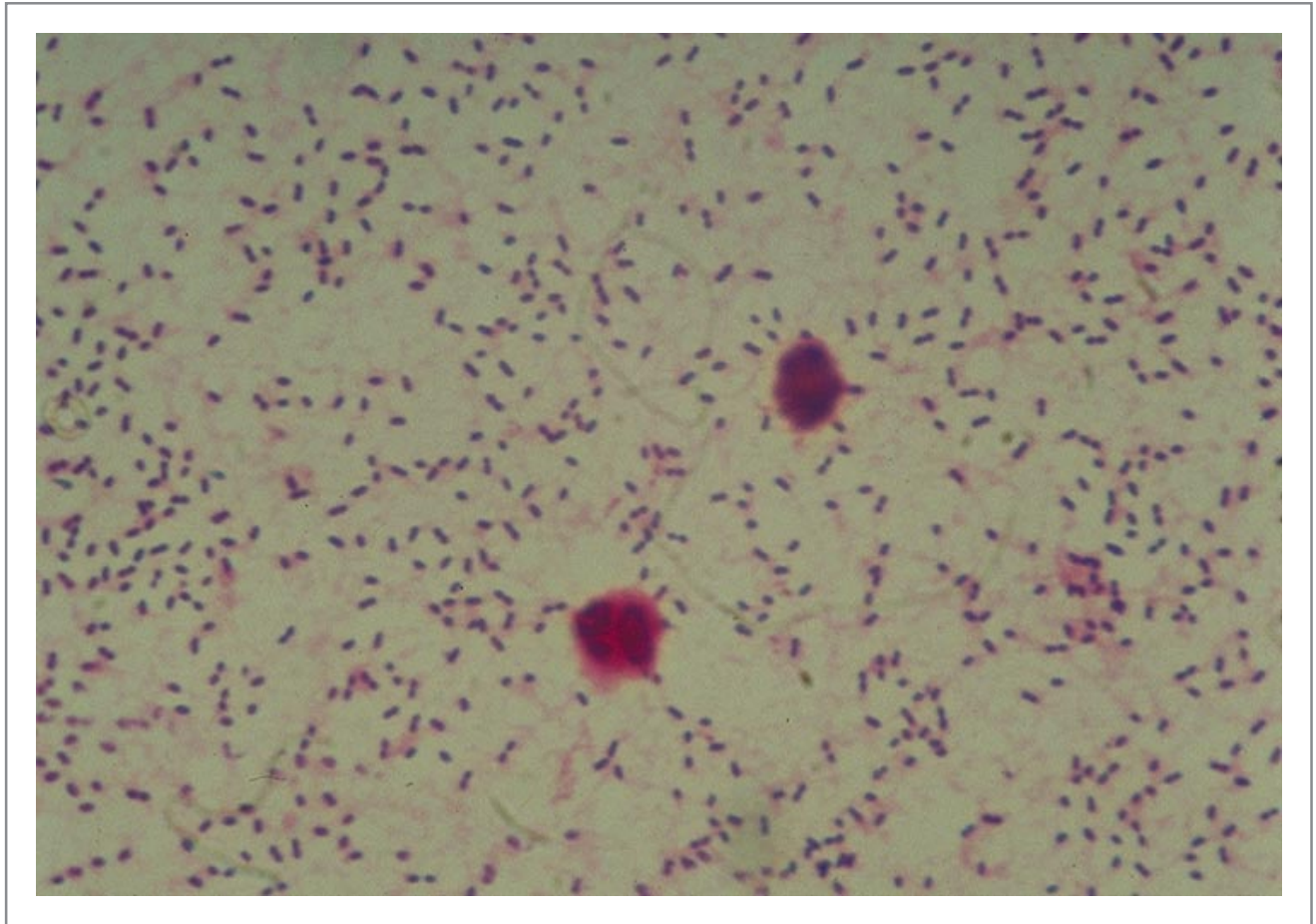


Figure 9b. Gram stain of cerebrospinal fluid: *S. pneumoniae*: gram-positive diplococci. Note that this slide represents a case where an exceptionally large number of organisms are present.

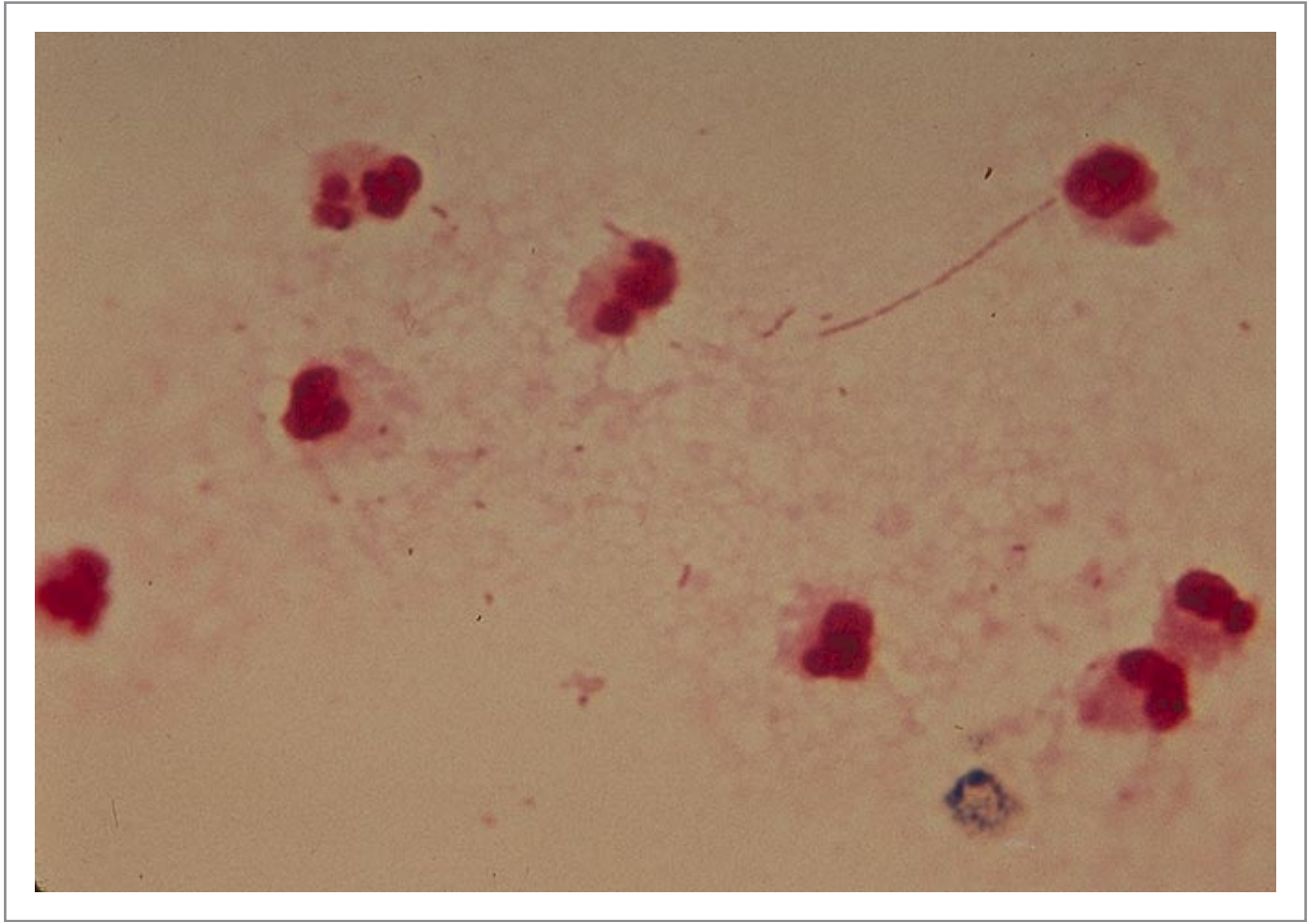


Figure 9c. Gram stain of cerebrospinal fluid: *H. influenzae*: gram-negative pleomorphic coccobacilli.

Moreover, *H. influenzae* appears as large, flat, colorless-to-grey opaque colonies (Figure 10). No haemolysis or discoloration of the medium is apparent.

On BAP, young colonies of *N. meningitidis* are round, smooth, moist, glistening and convex, with an entire edge. Some colonies appear to coalesce with their neighbours. Growth is greyish and unpigmented. Older cultures become more opaquely grey and sometimes cause the underlying agar to turn dark. Well-separated colonies can grow from about 1 mm in diameter in 18 hours to as large as 4 mm, with a somewhat undulating edge, after several days (Figure 11).

S. pneumoniae appears as small, greyish, moist (sometimes mucoid), watery colonies with a greenish zone of alpha-haemolysis surrounding them on BAP (Figure 12) and CAP. Young pneumococcal colonies appear raised, similar to viridans streptococci. However, once the culture ages 24 hours to 48 hours, the colony becomes flattened, and the central portion becomes depressed. Thus, a microscope (30x-50x) or a 3x hand lens can be a useful tool in differentiating pneumococci from alpha-haemolytic viridans streptococci. Presumptive identification can be made on the basis of the growth on BAP and CAP, and on the basis of the microscopic morphology of the organisms (Figures 13 and 14).



Figure 10. On chocolate agar plate, *H. influenzae* appear as large colorless to gray, opaque colonies with no discoloration of the surrounding medium.

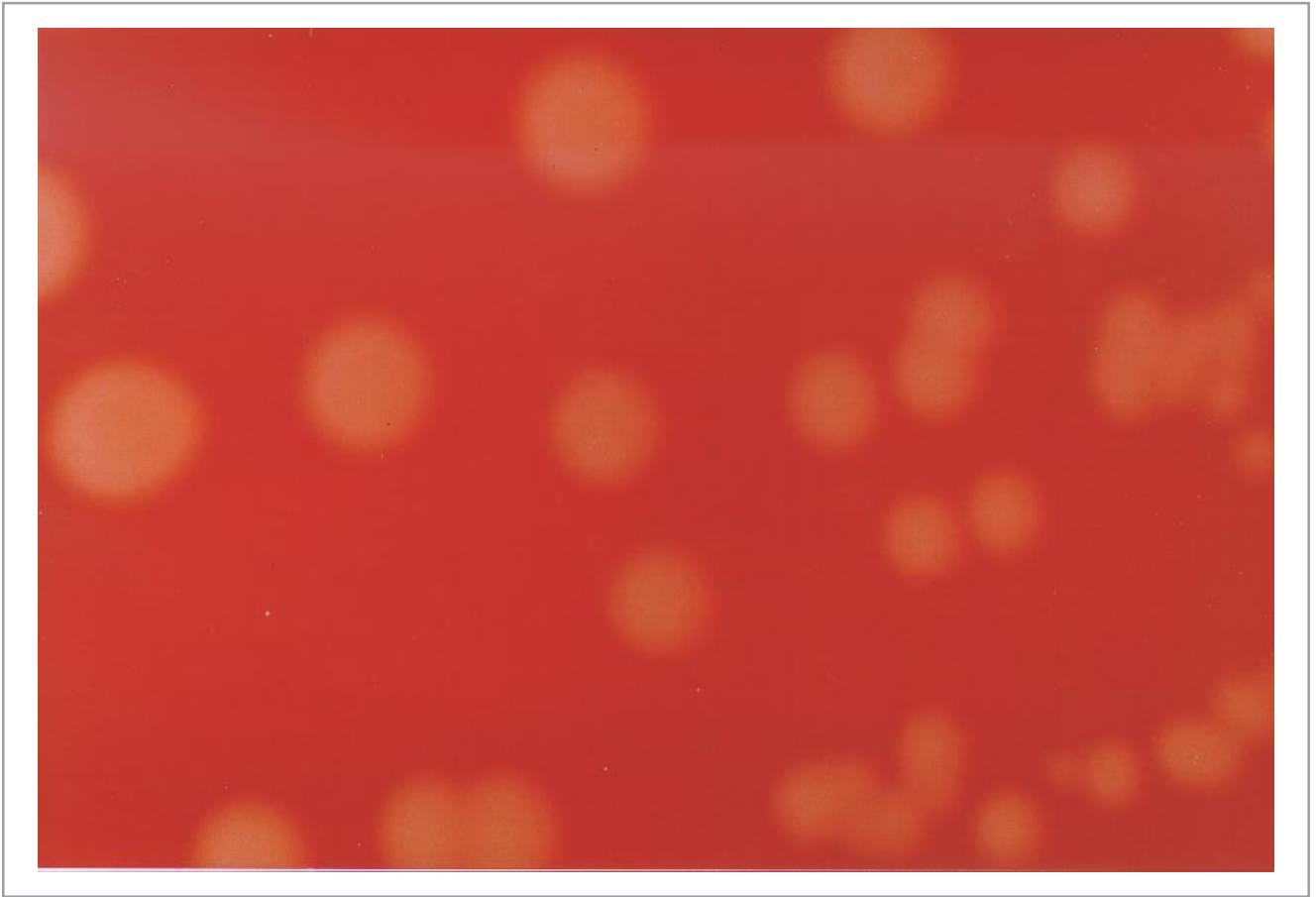


Figure 11. Overnight growth of *N. meningitidis* on blood agar plate appears as round, moist, glistening and convex colonies.

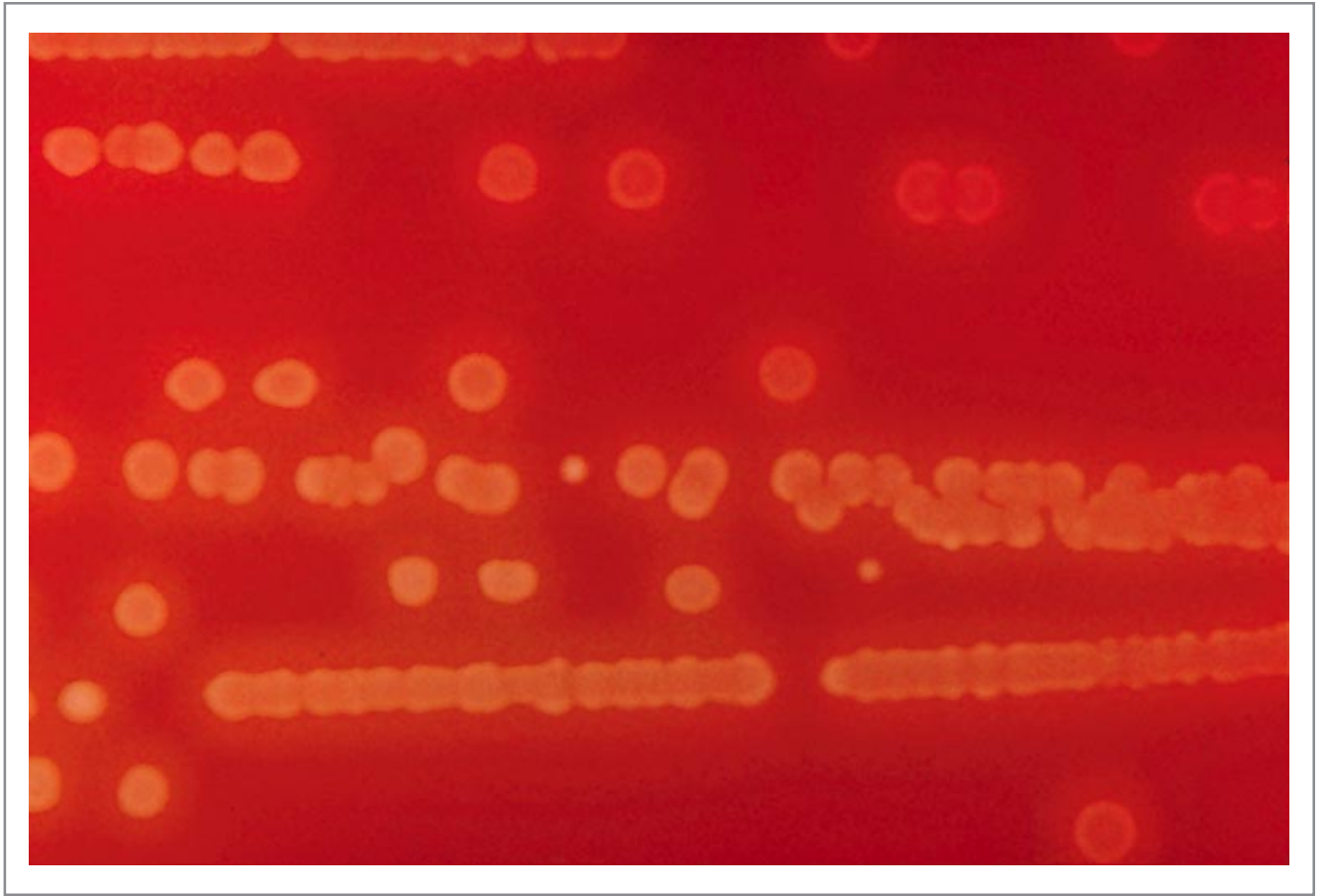


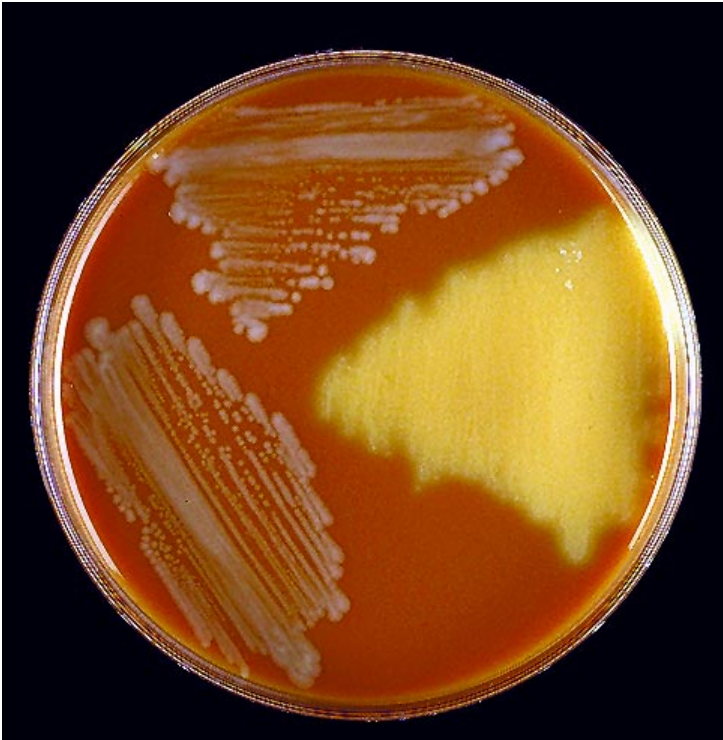
Figure 12. *S. pneumoniae* appear as small grayish mucoid (watery) colonies with a greenish zone of alpha-hemolysis surrounding them on the blood agar plate.

Growth on		Gram stain	Presumptive identification
CAP	BAP (Sheep)		
+	+	Gram-negative diplococci	<i>N. meningitidis</i>
+	+	Gram-positive diplococci	<i>S. pneumoniae</i>
+	—	Gram-negative pleomorphic coccobacilli	<i>H. influenzae</i>

Figure 13. Presumptive identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*.



(a) Growth on blood agar plate.



(b) Growth on chocolate agar plate.

Figure 14. *N. meningitidis* (left), *S. pneumoniae* (right), and *H. influenzae* (top):
(a) growth on blood agar plate and (b) growth on chocolate agar plate.

V. Identification of *N. meningitidis*

The following steps are recommended to confirm the identity of cultures that morphologically appear to be *N. meningitidis* (Figure 15). The best results are obtained with day-old cultures. Always check for purity of the growth by performing a Gram stain (*N. meningitidis* is a Gram-negative, coffee-bean-shaped diplococcus). When necessary, make subcultures to ensure purity. From growth on a BAP, perform Kovac's oxidase test, and then identify the serogroup. Finally, confirm the results with carbohydrate (sugar) reactions.

A. Kovac's Oxidase Test

The oxidase test determines the presence of cytochrome oxidase. The reagent tetramethyl-p-phenylenediamine hydrochloride is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain.

Preparation of 1% Oxidase Reagent from Powder

To prevent deterioration of stock oxidase-reagent powder, store in a tightly closed bottle in a desiccator kept in a cool dark area. Prepare 10 ml of a 1.0% tetramethyl-p-phenylenediamine hydrochloride solution in distilled water. Dispense the reagent in 1-ml aliquots and store frozen at -20°C . For use, thaw a 1-ml vial and wet as many strips of filter paper as possible on a nonporous surface (i.e. Petri dish, glass plate). Let the strips dry in air. When the strips are completely dry, place them in a tightly capped tube/bottle and refrigerate at 4°C . Use as needed.

Note: Oxidase reagent is intended only for in vitro diagnostic use. Avoid contact with the eyes and skin. It can cause irritation. In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes.

Performance of the Test

Using a platinum inoculating loop, a disposable plastic loop, or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto a treated strip of filter paper (Figure 16). Do not use a Nichrome loop, since it may produce a false-positive reaction.

Reading Test Results

Positive reactions will develop within 10 seconds in the form of a purple colour. Delayed reactions are unlikely with *N. meningitidis*. This test aids in the recognition of *N. meningitidis*, but other members of the genus *Neisseria*, as well as unrelated bacterial species, may also give a positive reaction.

B. Identification of the *N. meningitidis* Serogroup

Twelve serogroups, based on capsular polysaccharides, are currently recognized: A, B, C, H, I, K, L, W135, X, Y, Z, and Z' (29E).

Note: serogroup D is no longer recognized.

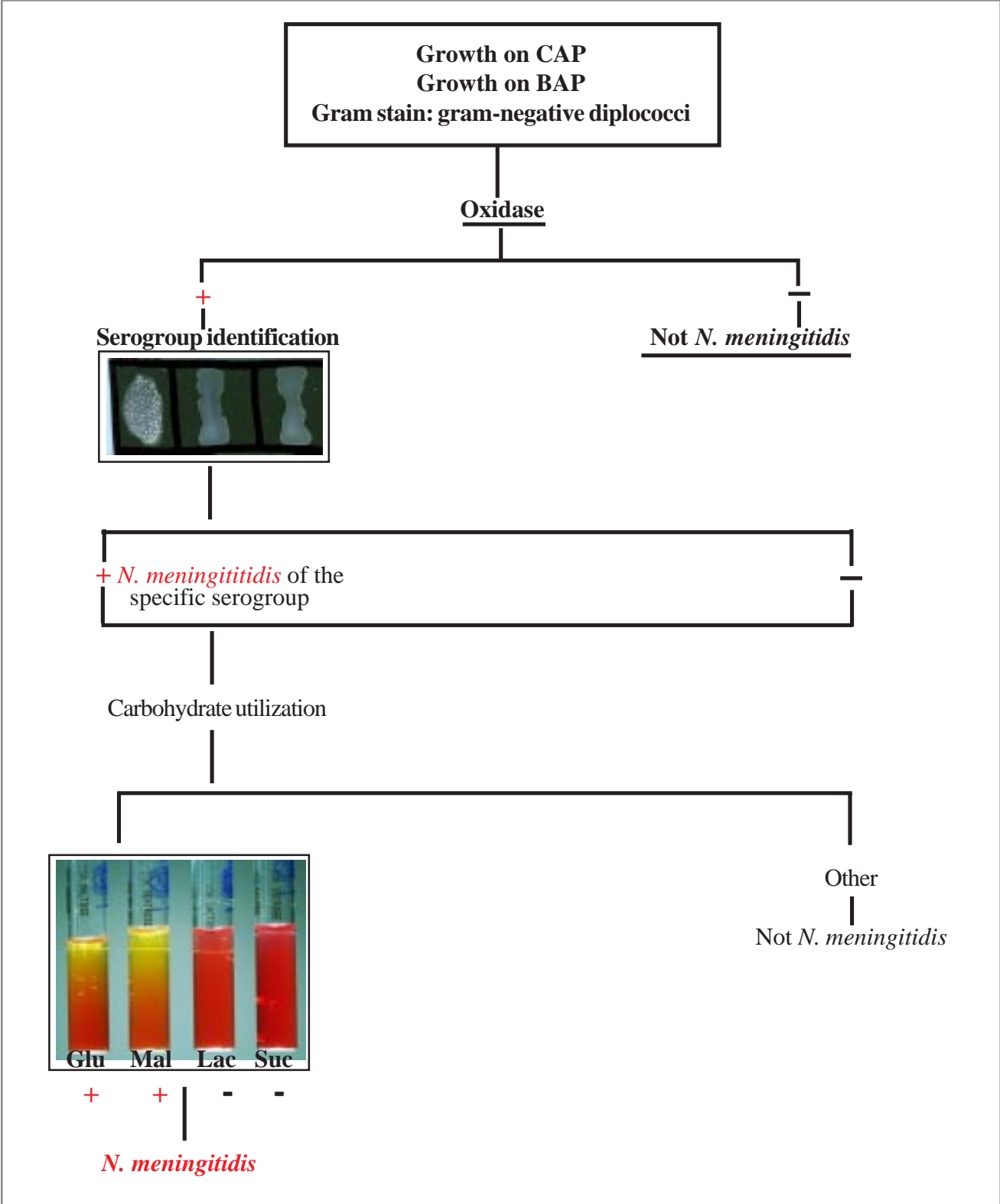


Figure 15. Identification of *N. meningitidis*.

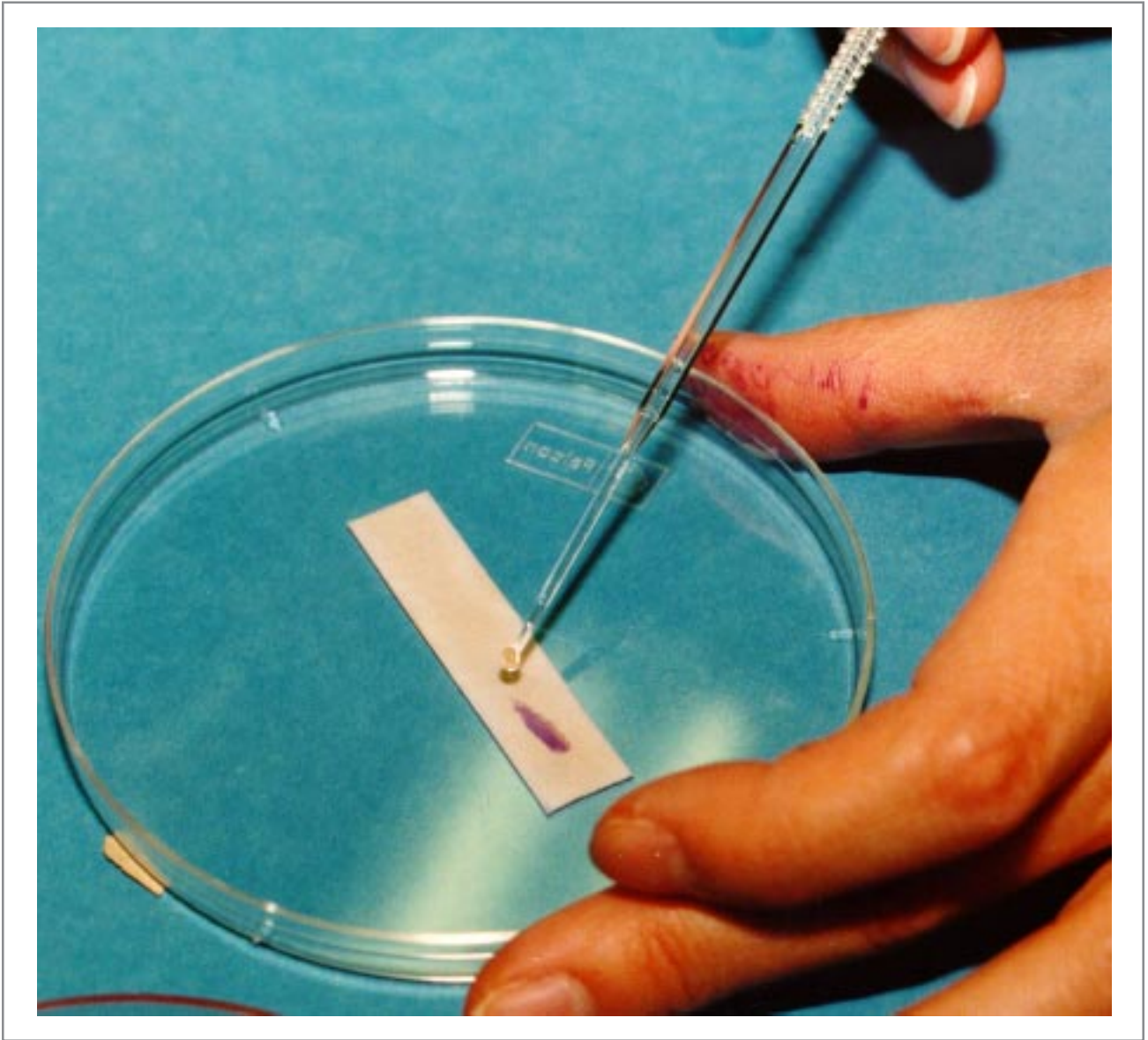


Figure 16. Kovac's oxidase test - positive reaction.

Groups A, B, C, W135 and Y are the most common causes of meningitis. Serogroup A is the most common cause of epidemics in Africa and Asia, followed by serogroup C. Grouping antisera are available commercially.

Performance of the Test

- (a) Clean a glass slide with alcohol. Divide the slide into three 10x4-mm sections with a wax pencil or other marker.
- (b) Place 10 µl of 0.5% formalinized saline near the bottom of each of three sections. Collect a portion of the growth from the surface of the BAP with a sterile inoculating loop, needle, sterile applicator stick or toothpick. Make a moderately milky suspension of the culture being tested in each drop of saline.
Note: For safety reasons, it is recommended to use formalin-killed meningococcal suspensions rather than saline suspensions of living organisms. However, formalin is a carcinogen and must be stored and handled with great care. Alternatively, work under a safety hood.
- (c) In the upper portion of each glass section, add 10 µl each of the antiserum of choice and saline/phosphate-buffered saline (PBS) (unformalinized).
Note: In Africa, testing with A and C antisera with a saline control to detect nonspecific autoagglutination should be adequate to characterize most isolates serologically. Strains reacting negatively with A and C antisera should then be tested with other available antisera, particularly Y, W135 and B.
- (d) Mix each antiserum and saline with the respective culture suspension and rock the slide for 1 to 2 minutes (time may vary depending on the manufacturer of the antisera) under a bright light and over a black background.

Reading the Test Results

Agglutination should occur with only one of the antisera and not with saline (Figure 17). Agglutination in saline would characterize the culture as autoagglutinable. Agglutination with several serum samples in the absence of agglutination in saline would characterize the culture as rough. No agglutination with any of the antisera or the saline would characterize the strain as non-groupable. These results are rare with fresh case isolates, but they do happen occasionally. Store antisera and saline/PBS in the refrigerator at 4 °C when not in use.

C. Carbohydrate Utilization by *N. meningitidis* - Cystine Trypticase Agar Method

Carbohydrate utilization tests are used to further validate the identification of a strain as *N. meningitidis*. Various carbohydrates are added to the cystine trypticase agar base to a final concentration of 1%. To confirm a culture as *N. meningitidis*, a set of four tubes, each containing a sugar (glucose, maltose, lactose, and sucrose) is used. Members of *Neisseria* spp. produce acid from carbohydrates by oxidation, not fermentation. *N. meningitidis* oxidizes glucose and maltose, but not lactose and sucrose. A phenol red indicator is included in the medium; it is a sensitive indicator that develops a yellow colour in the presence of acid, at a pH of 6.8 or less.

Performance of the Test

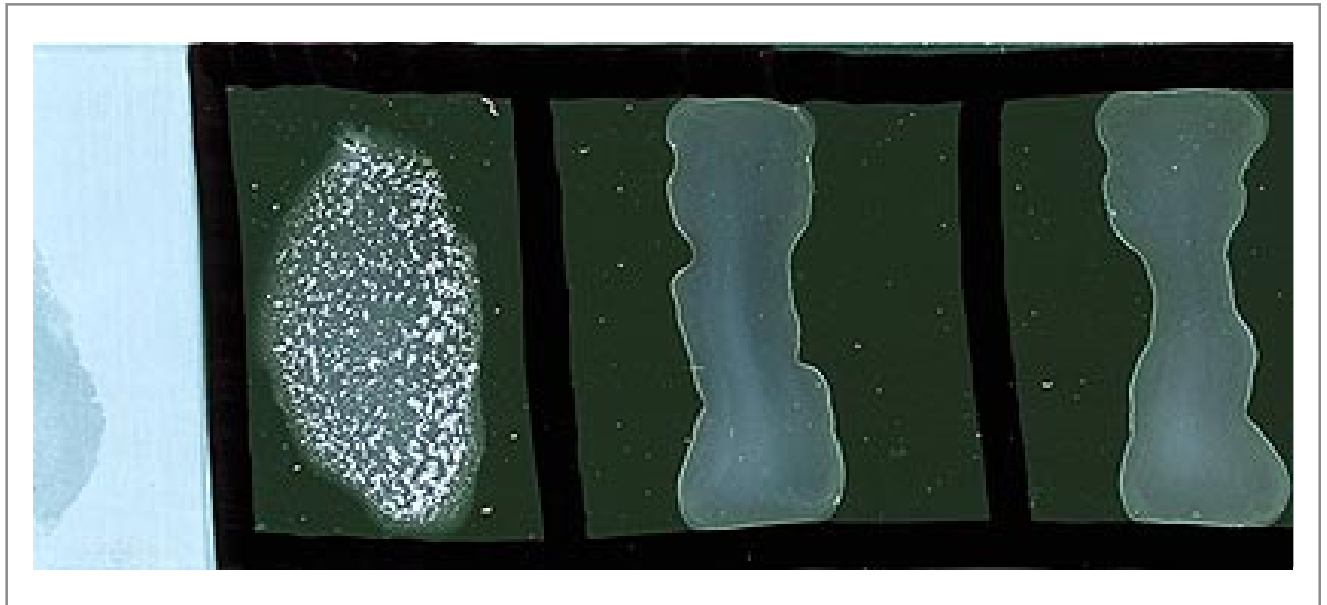


Figure 17. Agglutination, with clearing of the liquid, occurs when a suspension of the isolate is mixed with its homologous antiserum (left). A negative reaction, which should occur with heterologous antiserum (center) and with saline (right), remains smooth and turbid.

- (a) With an inoculating needle, take up a small amount of growth from an overnight culture on BAP or CAP.
- (b) Stab the inoculum several times into the upper 10 mm of medium. Use a separate needle, or flame the needle, before inoculating each carbohydrate to be tested.
- (c) Fasten caps of tubes **tightly** and place in a 35 °C incubator (without CO₂). Incubate for at least 72 hours before discarding as negative.

Reading the Test Results

Development of visible turbidity and a yellow colour in the upper portion of the medium indicates growth and the production of acid and is interpreted as a positive test (Figure 18). Although reactions may occur as early as 24 hours after inoculation, some reactions are delayed, and negative results should not be interpreted before 72 hours of incubation (Table 1).

D. Commercial Identification Kits

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Neisseria* spp. These systems may occasionally require supplemental tests, and additional characteristics, such as microscopic and colony morphology, must be considered. Generally, each system is self-contained, but addition of one or more reagents to complete certain reactions may be necessary. Follow the manufacturer's instructions precisely when using these kits. For detailed instructions and use of appropriate control strains also consult the Clinical Microbiology Procedures Handbook. Alternatively, the rapid sugar utilization tests may be used.

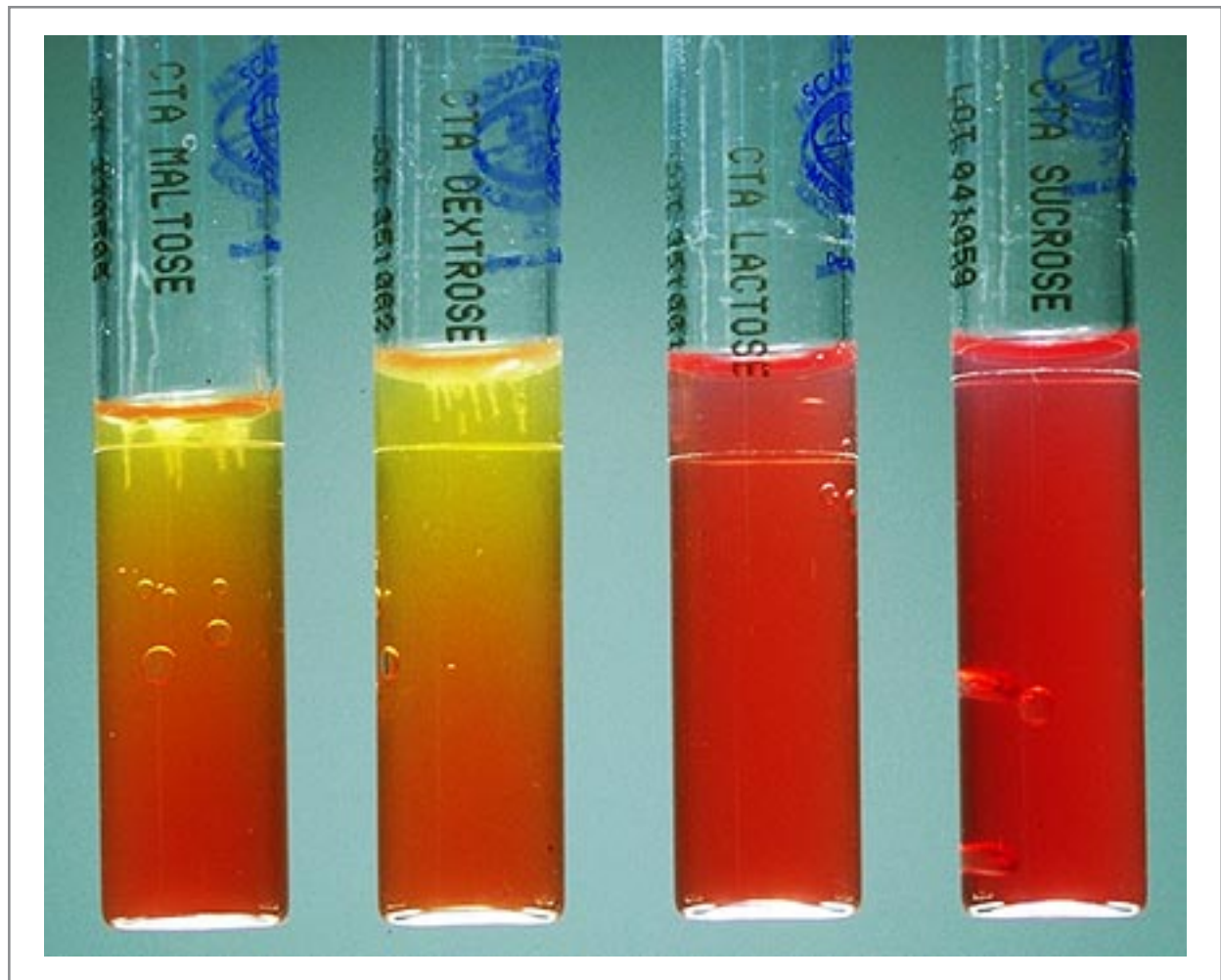


Figure 18. Cystine trypticase agar-sugar reactions differentiating *N. meningitidis* from other *Neisseria* species. Acid production (yellow color) shows oxidative utilization of dextrose and maltose with no utilization of lactose and sucrose.

**Table 1: Carbohydrate Utilization by Some Species
of the Genus *Neisseria* and *Moraxella***

Species	Acid Production from:			
	Glucose	Maltose	Lactose	Sucrose
<i>Neisseria meningitidis</i>	+	+	-	-
<i>Neisseria gonorrhoeae</i>	+	-	-	-
<i>Neisseria sicca</i>	+	+	-	+
<i>Neisseria lactamica</i>	+	+	+	-
<i>Moraxella catarrhalis</i>	-	-	-	-

VI. Identification of *S. pneumoniae*

S. pneumoniae appear as small, greyish, mucoid (watery) colonies surrounded by a greenish zone of alpha-haemolysis on BAP and CAP. A 3x hand lens or a microscope (30x-50x) is a useful aid in differentiating pneumococci from alpha-haemolytic viridans streptococci. Both types of colonies appear raised when young. However, after 24-48 hours, the centre of pneumococcal colonies becomes depressed whereas viridans streptococcal colonies retain their raised appearance. Differentiation between *S. pneumoniae* and viridans streptococci is accomplished by optochin and bile solubility testing (Figure 19). For optimal results it is recommended that plates for all assays be incubated in a CO₂ atmosphere.

A. Susceptibility to Optochin

Presumptive identification of *S. pneumoniae* is made by determining the susceptibility of the strain to optochin.

Performance of the Test

- (a) Touch the suspect alpha-haemolytic colony with a sterile bacteriological loop and streak onto a BAP, as shown in Figure 7.
- (b) Aseptically place an optochin or “p” disk with a diameter of 6 mm (containing 5 µg ethylhydrocupreine) on the end of the streak where the wire loop was first placed. Three to four colonies may be tested on the same plate.
- (c) Incubate the plates in a CO₂ incubator or candle-jar at 35 °C for 18-24 hours.

Reading the Test Results

In Figure 20, the strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the centre and lower streaks are susceptible to optochin and appear to be pneumococci. Alpha-haemolytic strains with a zone of inhibition of growth greater than 14 mm in diameter are pneumococci. Alpha-haemolytic strains with no zones of inhibition are viridans streptococci. Alpha-haemolytic strains with zones of inhibition ranging between 9 and 13 mm should be tested for bile solubility.

B. Bile Solubility Test

Performance of the Tests

- (a) Take a loop of the strain from the growth on a BAP and make a suspension in 0.5 ml of sterile saline. If there is sufficient growth on the optochin test plate, the suspension can be made with the cells taken from the single streak. When there is insufficient growth to make a satisfactory suspension, a fresh culture on blood agar will have to be made. The suspension of cells should be equal to that of a 0.5 McFarland density standard (Annex C5).

- (b) Divide the suspension into two equal amounts (0.25 ml per tube), and add 0.25 ml of saline to one tube and 0.25 ml of 2% deoxycholate (bile salts) to the other. Shake the tubes gently and incubate them at 35 °C-37 °C for up to 2 hours.
- (c) Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, as shown in Figure 21, is a positive result.

Alternatively, in a plate method for testing bile solubility, a drop of solution of 10% sodium deoxycholate is placed directly on a colony of the strain to be tested. The plate can be kept at room temperature or placed right side up and on a level surface in an aerobic incubator at 35 °C for approximately 15 minutes or until reagent dries. Pneumococcal colonies will disappear or be flattened, while bile-resistant streptococcal colonies will be unaffected. It is essential to use a freshly prepared plate for this test.

Interpreting the Test Results

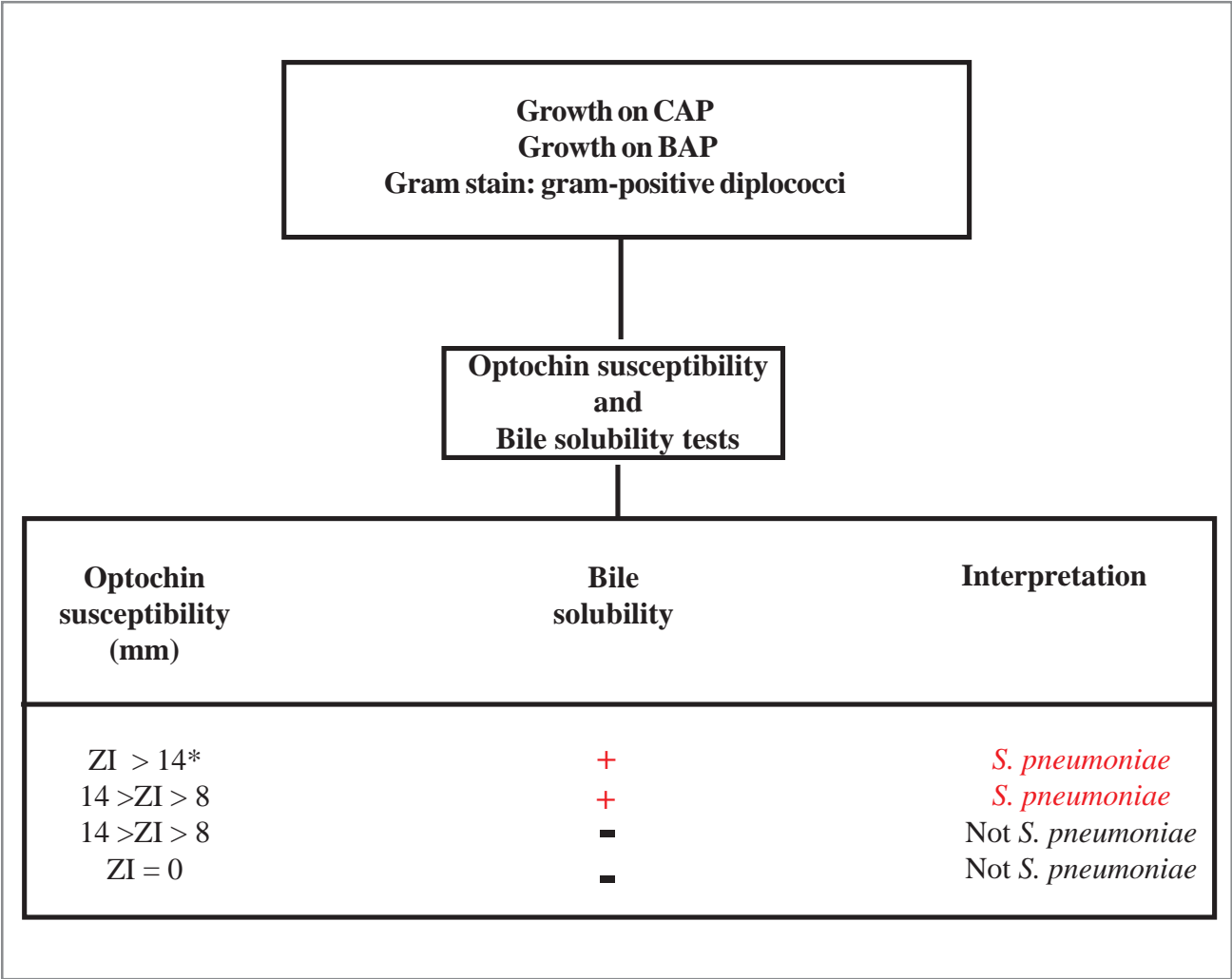
Usually, the most accurate and convenient identification of pneumococci, based on the optochin and bile solubility tests, is the following:

- (a) A strain showing a zone of inhibition by optochin of 14 mm or more is a pneumococcus.
- (b) A strain showing a smaller but definite zone of inhibition by optochin and is bile soluble is also a pneumococcus.
- (c) A strain with a small zone of inhibition by optochin that is not bile soluble is not a pneumococcus.
- (d) Strains with no zones of inhibition by optochin are not pneumococci (Figure 19).

C. Slide Agglutination Test

Commercially available slide agglutination tests can help identify colony growth from BAP as pneumococci (e.g. Slidex Pneumo [Vitek Systems, Inc., Hazelwood, MO] or Pneumoslides [BBL Microbiology Systems, Cockeysville, MD]). Follow the manufacturer's instructions precisely when using these tests.

Alternatively, in a plate method for testing bile solubility, a drop of solution of 10% sodium deoxycholate is placed directly on a colony of the strain to be tested. The plate can be kept at room temperature or placed right side up and on a level surface in an aerobic incubator at 35 °C for approximately 15 minutes or until the reagent dries. Pneumococcal colonies will disappear or be flattened, while bile-resistant streptococcal colonies will be unaffected. It is essential to use a freshly prepared plate for this test.



Note: ZI = Zone of inhibition for BBL optochin disks. When using any other brand of optochin disks, follow the instructions for the interpretation of zones of inhibition, as specified by that manufacturer.

*95% of clinical *S. pneumoniae* isolates react in this manner

Figure 19. Identification of *S. pneumoniae*.

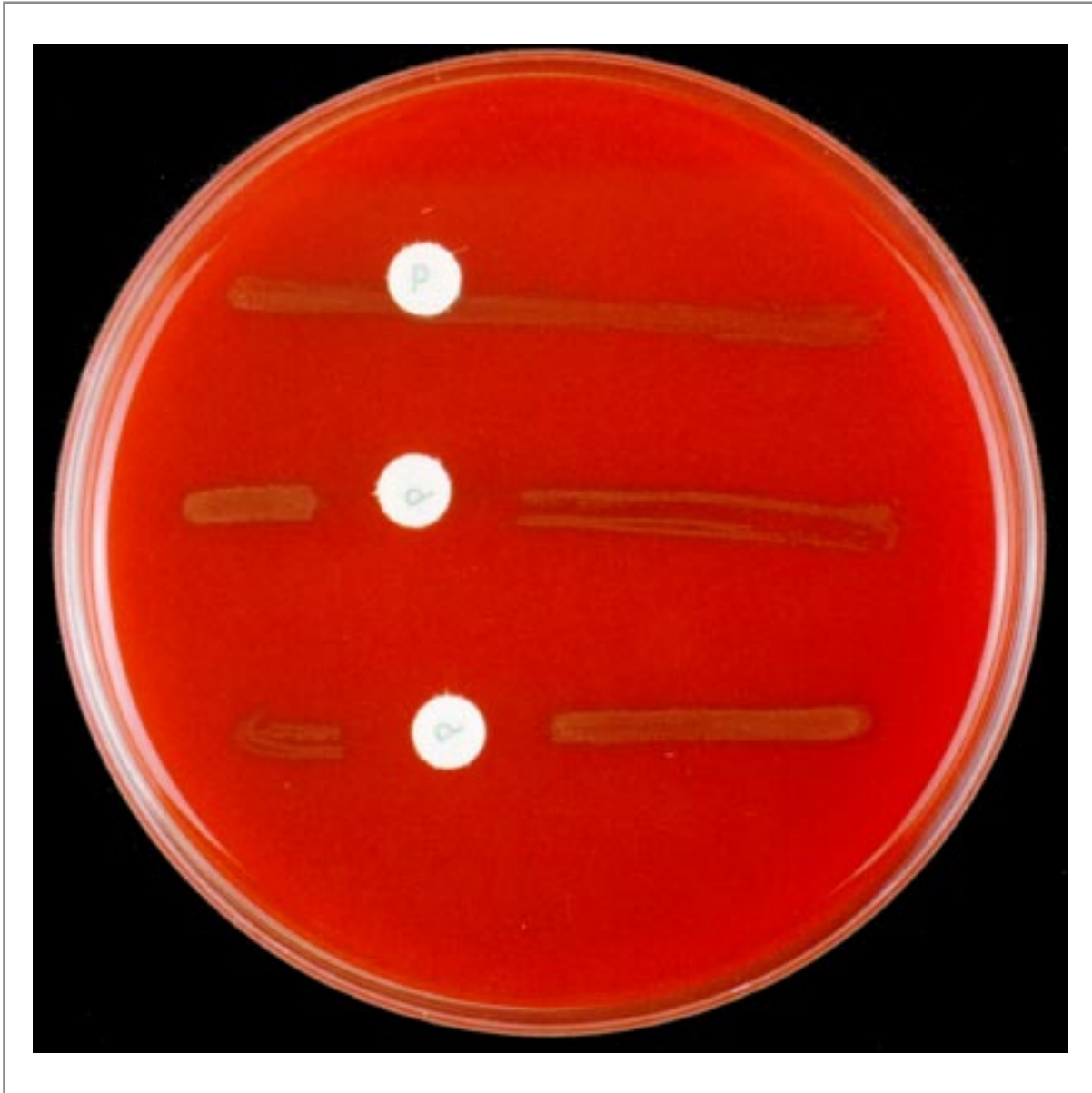


Figure 20. Optochin susceptibility test for identification of *S. pneumoniae*. The strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the center and lower streaks are susceptible to optochin and appear to be pneumococci.



Figure 21. The bile solubility test for two different strains. Strain 1 is not *S. pneumoniae* as both tubes are turbid. There is a slight decrease in turbidity in the tube containing bile salts for strain 1 (2nd tube from the left) but the tube is almost as turbid as the control tube (1st tube on the left). Strain 2 is *S. pneumoniae*. Note that the tube on the far right is clear, all the turbidity due to the cells has disappeared and the cells have lysed; by contrast the control tube (3rd tube from the left) is still very turbid.

VII. Identification of *H. influenzae*

Small, Gram-negative bacilli or coccobacilli which require X and V factors, grow on CAP and not on BAP, have a pungent indol smell, and do not group with meningococcal antisera, may be *H. influenzae* (Figure 22). In the absence of vaccination, almost all cases of meningitis caused by *H. influenzae* are serotype b.

A. Identification of the *H. influenzae* Serotype

Performance of the test is similar to that described for *N. meningitidis* except for the choice of antisera. Suspected Hib isolates should be tested against *H. influenzae* type b antiserum, an antiserum to one of the other groups, and saline. A strongly positive reaction with b antiserum and no reactivity with an antiserum to one of the other groups and saline is presumptive evidence of Hib. If the isolate is non-reactive with the b antiserum, test it with a polyvalent antiserum. If positive, the isolate must be tested with three maining antisera (a, c, d, e, f) to determine its serotype. If negative, it is probably non-typable. Determination of X and V factor requirements is necessary to confirm the identity of the isolate as *H. influenzae* or another *Haemophilus* species.

Performance of the Test

- (a) Make a milky suspension of cells from an overnight culture on the CAP or the *Haemophilus* ID plate in 10 µl of 0.5% formalinized saline.
- (b) For the agglutination reaction, transfer a loopful (5 µl) of the cell suspension to an ethanol-washed slide divided into three sections.
- (c) To each section, respectively, add an equal volume of Hib antiserum, a different type specific antiserum, and saline.
- (d) Mix with a toothpick and gently rock the slide for up to a minute.

Reading of Test Results

Only strong agglutination reactions are read as positive. In a strong reaction, all the bacterial cells will be clumped and the suspension fluid will appear clear. When a strain reacts with more than one antiserum, the result is recorded as non-typable.

Note: *Haemophilus* can also be serotyped by using the Quellung reaction, as described for the serotyping of *S. pneumoniae* (Annex D).

B. Identification of X and V Factor Requirements

B1. X, V and XV Paper Disks and Strips

H. influenzae is a fastidious organism requiring media containing haemin (X factor) and nicotinamide adenine dinucleotide (NAD, V factor). Growth occurs on CAP because of haemin released during the heating process in the preparation of chocolate agar. Haemin is available from non-haemolyzed as well as haemolyzed cells. Alternatively, NAD is added as an IsoVitalax or Vitox component. *H. influenzae* is identified on the basis of its growth requirements for X and V factors (Table 2).

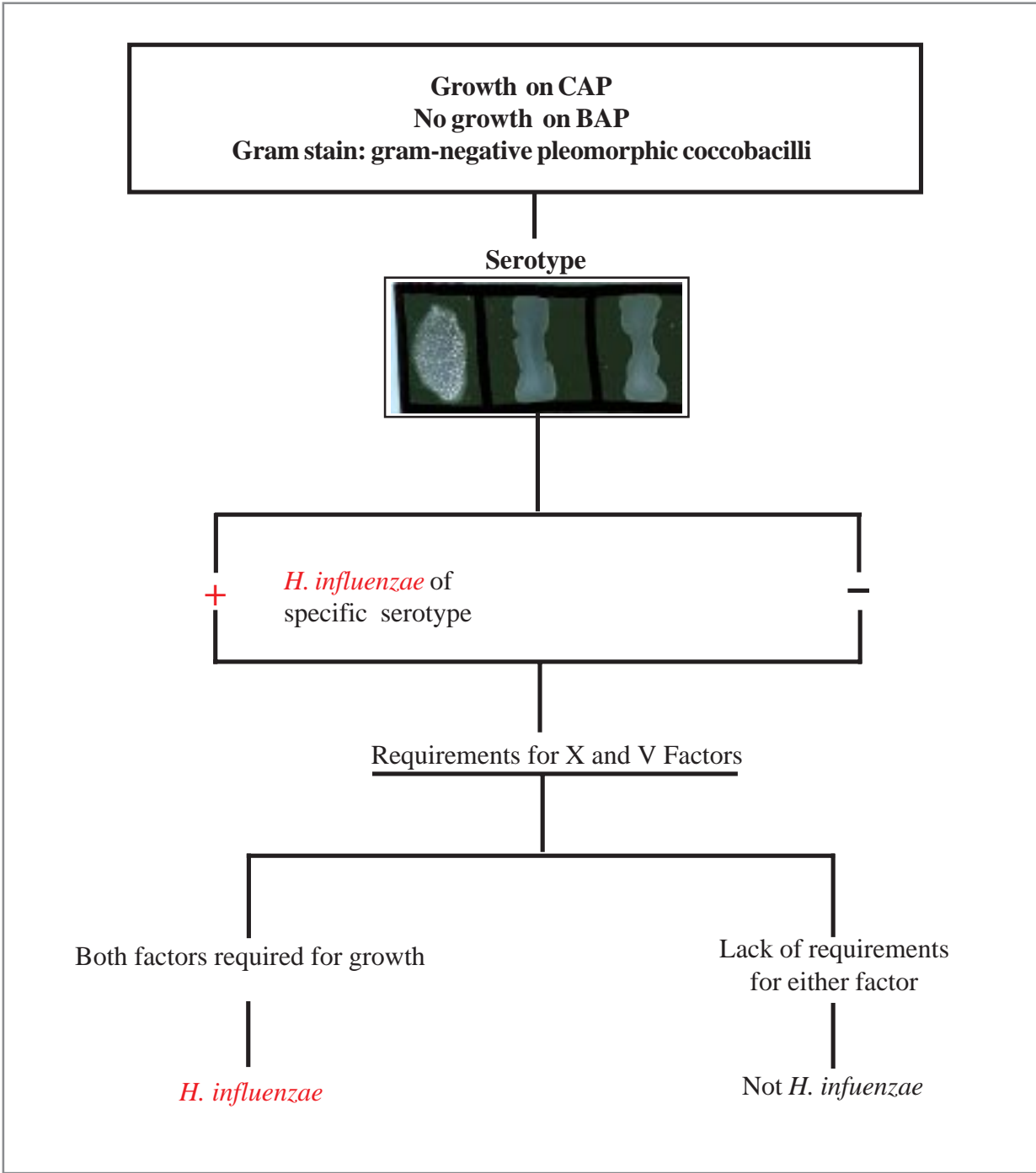


Figure 22. Identification of *H. influenzae*.

Performance of the Test

- (a) Prepare a heavy suspension of cells (No. 1 McFarland) from a primary isolation plate in a suitable broth (trypticase soy, heart infusion, or peptone water). If the primary isolation plate contains insufficient growth or is contaminated, make a subculture on a CAP. Caution should be used in preparation of the broth to avoid transfer of media to the broth; even the smallest sample of agar will affect the test and may lead to misidentification of the bacteria.
- (b) Inoculate a heart infusion or tryptic soy agar plate. A sterile swab of the suspension is streaked over one-half of the plate, and paper strips or disks containing X, V, and XV factors are placed on the inoculated plate after the inoculum has dried. When two bacterial strains are tested on the same plate, as shown in Figure 23, the disks must be placed in the exact manner shown.

Reading the Test Results

H. influenzae will grow only around the disk containing both X and V factors, as shown in Figure 23 on the upper half of the plate.

Alternatively, the porphyrin test of Kilian can be used. This determines the X-factor requirement of the isolate while avoiding the problem of X-factor carryover from primary culture media and X-factor contamination of test media (Manual of Clinical Microbiology).

B2. *Haemophilus* ID “Quad” Plates

These plates are another, although more expensive, method for determining factor requirements of *Haemophilus* isolates (Figure 24). This agar plate is divided into four compartments. One quadrant includes medium containing haemin (X factor) (upper left). One quadrant includes medium containing NAD (V factor) (lower left). Another quadrant contains medium that includes both X and V factors (upper right). The fourth quadrant contains heart infusion agar or blood agar base with 5% horse blood (lower right) for differentiating

H. haemolyticus, an oral species requiring X and V factors, from *H. influenzae*.

Performance of the Test

- (a) Inoculate the *Haemophilus* ID plate by suspending the growth from a young, pure culture of suspected *Haemophilus* in TSB or distilled water to approximately the turbidity of 0.5 McFarland standard. Using a bacteriological loop, streak one loopful of this suspension on each quadrant of the plate, beginning with the V quadrant and ending with the blood quadrant. Streak the entire quadrant, starting at the periphery and streaking toward the centre of the plate. Stab into the blood agar for detection of weak haemolysis.
- (b) Incubate at 35 °C in a candle-jar or CO₂ incubator for 18-24 hours.

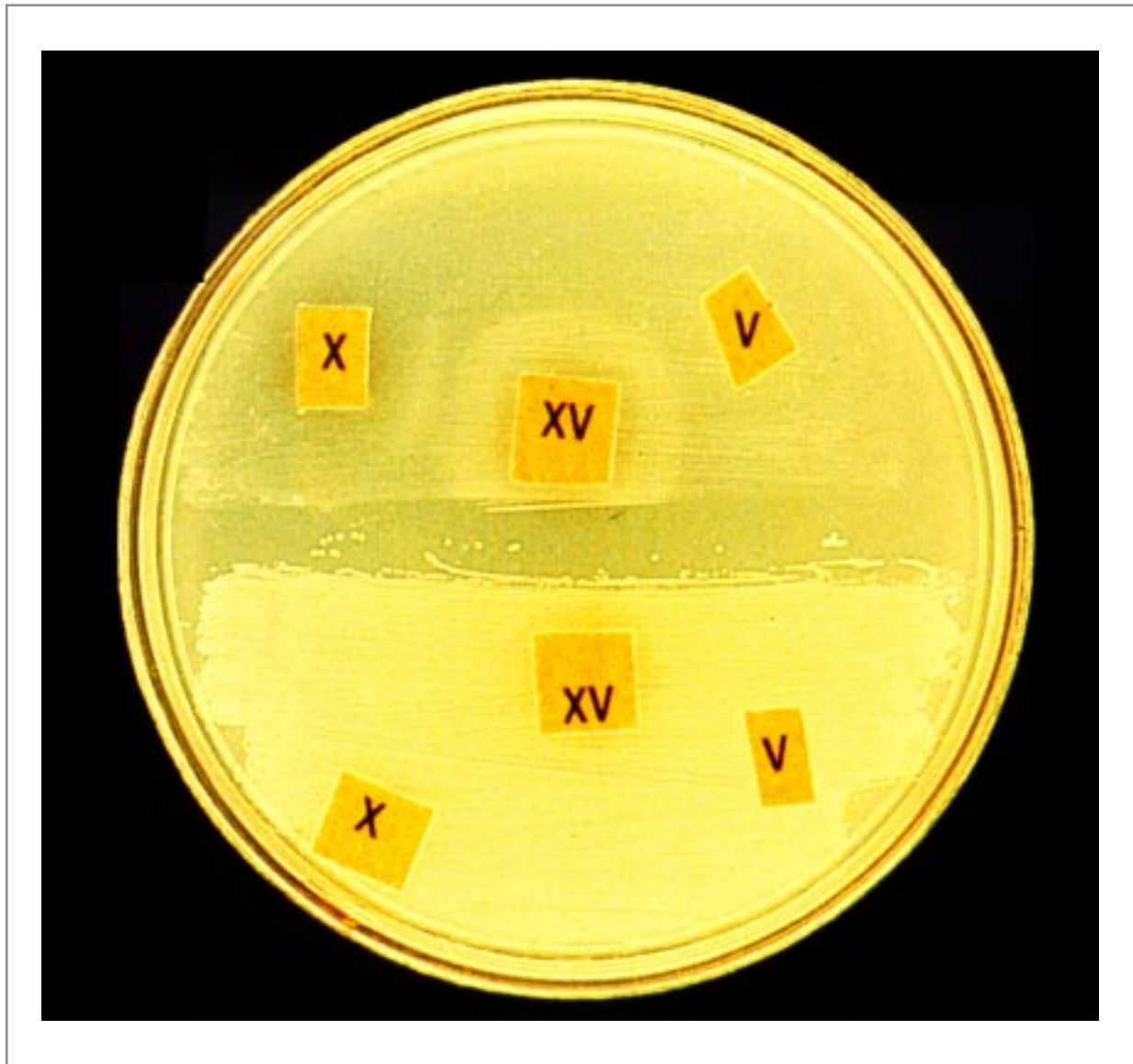


Figure 23. Growth factor requirements. *H. influenzae* will grow only around the disk containing both X and V factors.

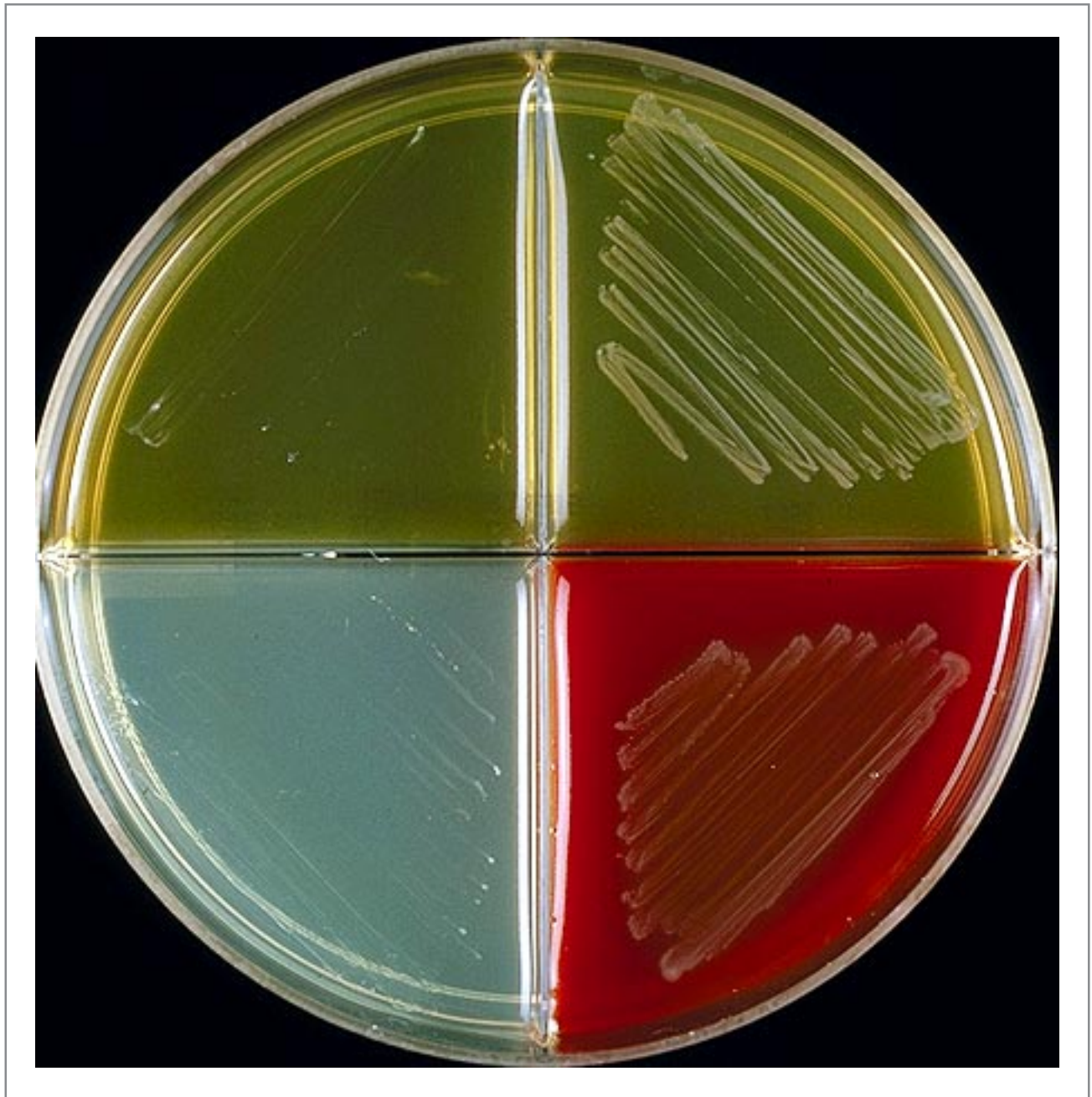


Figure 24. Growth factor requirements for *H. influenzae*: *Haemophilus* quad ID plate. This agar plate is divided into four compartments. One quadrant includes medium containing haemin (X factor) (upper left). One quadrant includes medium containing nicotinamide dinucleotide (V factor) (lower left). Another quadrant contains medium that includes both X and V factors (upper right). The fourth quadrant contains heart infusion agar with 5% horse blood (lower right).

Table 2: Identification of *Haemophilus* Species by their Growth Requirements

Species	X- and V- Factor Requirements		β-haemolysis on Rabbit
	X	V	Blood Agar
<i>H. influenzae</i>	+	+	-
<i>H. parainfluenzae</i>	-	+	-
<i>H. haemolyticus</i>	+	+	+
<i>H. parahaemolyticus</i>	-	+	+
<i>H. aphrophilus</i>	+	-	-
<i>H. paraphrophilus</i> *	-	+	-

**H. paraphrophilus* is ornithine negative, whereas *H. parainfluenzae* is positive.

Reading the Test Results

After incubation, examine the blood section for haemolysis and the other sections for growth (Figure 24). An organism growing on both the V and X quadrant requires neither, and thus is not a *Haemophilus*. Very light background growth on the V section may be seen with some X- and V-requiring *Haemophilus* strains. This should be disregarded.

VIII. Preservation and Transport of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*

To confirm the identification and to test antimicrobial susceptibility of bacteria isolated from cases of meningitis, it may be necessary to preserve and transport the strains to national, and/or WHO Reference Laboratories. *N. meningitidis*, *S. pneumoniae* and *H. influenzae* are fragile bacteria, and care must be employed to preserve and transport them.

A. Short-Term Storage

Viability during short-term storage (one week or less) is best if *S. pneumoniae* and *H. influenzae* are inoculated onto chocolate agar slants (with screw-capped tubes), incubated overnight at 35 °C, and then maintained at 4 °C. These bacterial species do not survive well in broth and survive only 3-4 days on primary agar plates. For *N. meningitidis*, solid screw-caps should be loosened during storage but permeable membrane screw-caps, which allow for an exchange of gases and are available commercially (Biomedical Polymers, Leominster, MA) should be used. An overlay of TSB may also be helpful, and may increase viability to 14 days. *N. meningitidis* slants should not be refrigerated.

B. Long-Term Storage

Long-term storage is best accomplished by either lyophilization or freezing. Lyophilization is the most convenient method of storage because lyophilized bacteria can be stored for long periods at 4 °C or -20 °C and should not be adversely affected by transport.

B1. Preservation by Lyophilization

- (a) Grow the *H. influenzae* on CAP and the *S. pneumoniae* and *N. meningitidis* on BAP or CAP. Incubate the plates in a CO₂ incubator or candle-jar for 18-20 hours at 35 °C. Inspect the plate for purity.
- (b) Harvest the growth from the plate with 1-2 ml of sterile skim milk. Place about 0.5 ml of suspension into a sterile ampoule or lyophilization vial. Several vials can be prepared from a single plate, if desired. Maintain sterility at all times during the preparation of the vial.
- (c) The cell suspension should be shell-frozen on the walls of the lyophilization vial. This is accomplished by one of two methods. Keep the lyophilization vial at -70 °C until just before the cell suspension is added. Add the cell suspension, and rapidly rotate the vial to freeze the suspension to the wall. Return the vial to the -70 °C freezer until ready to attach to the lyophilizer. If a -70 °C freezer is not available, a mixture of alcohol (95% ethanol) and dry ice can be prepared and used to shell-freeze the cell suspensions. Shell-freezing is accomplished by placing the cell suspension in the lyophilization vial and rotating the vial at a 45° to 60° angle in the alcohol-dry ice mixture.
- (d) Attach the vials to the lyophilizer. Follow the manufacturer's instructions since each instrument uses different types of apparatus. The time of lyophilization will depend

upon the number of vials being lyophilized and the capacity of the instrument. On an average machine, 4-5 hours are required to completely dry 10 to 20 small vials.

- (e) The vials are sealed at the end of the run. Seal the vials with a torch while they are still attached to the lyophilizer and under vacuum. The vials can be stored at 4 °C or at freezer temperatures after being sealed.

B2. Preservation by Freezing

- (a) Grow the *H. influenzae* on CAP and the *S. pneumoniae* and *N. meningitidis* on BAP or CAP. Incubate the plates in a CO₂ incubator or candle-jar for 18-20 hours at 35 °C. Inspect the plates for purity.
- (b) Harvest all of the growth from a plate with a sterile swab.
- (c) Dispense the growth in a 2-ml screw-capped (externally threaded) cryogenic vial, containing 1 ml of sterile defibrinated blood, by twirling the swab to release the organisms. Defibrinated sheep, horse, or rabbit blood can be used for all of these organisms. Human blood should not be used. Alternatives such as TSB with 15%-20% reagent grade glycerol, or Greaves solution can also be used. The vials should be cryogenic (able to withstand extreme cold).
- (d) Squeeze the excess blood from the swab by rotating it against the sides of the vial before carefully withdrawing it.
- (e) Discard the swab in disinfectant.
- (f) If possible, rapidly freeze the suspension in a bath of 95% alcohol and dry-ice pellets.
- (g) Place the vials in a nitrogen freezer (-120 °C) or a -70 °C freezer. A -20 °C freezer can be used, but some loss of viability can be expected. Freezers with automatic defrosters should never be used.

C. Transportation of Cultures

Lyophilized cultures and chocolate agar slant cultures can be transported without refrigeration. The cultures should be packaged according to the regulations specified in the WHO Laboratory Biosafety Manual. Each vial or tube should be individually wrapped before being enclosed in a metal container including absorbent material. This container is then inclosed in a cardboard shipping container. An address label and an etiological agent-warning label (EA label) are attached to the shipping container. No more than 50 ml of culture should be shipped in one package.

Individually wrapped, lyophilized culture vials can be transported in double-mailing containers. Cultures stored in the frozen state must be recultured, inoculated onto chocolate agar slants in screw-capped tubes, incubated overnight, and then packaged for transport as indicated above. *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* will usually survive for at least a week under these conditions.

C1. Transport in Silica Gel Packages

N. meningitidis, *S. pneumoniae* and *H. influenzae* can be successfully transported in silica gel packages, which are easy to handle and ship (Figure 25).

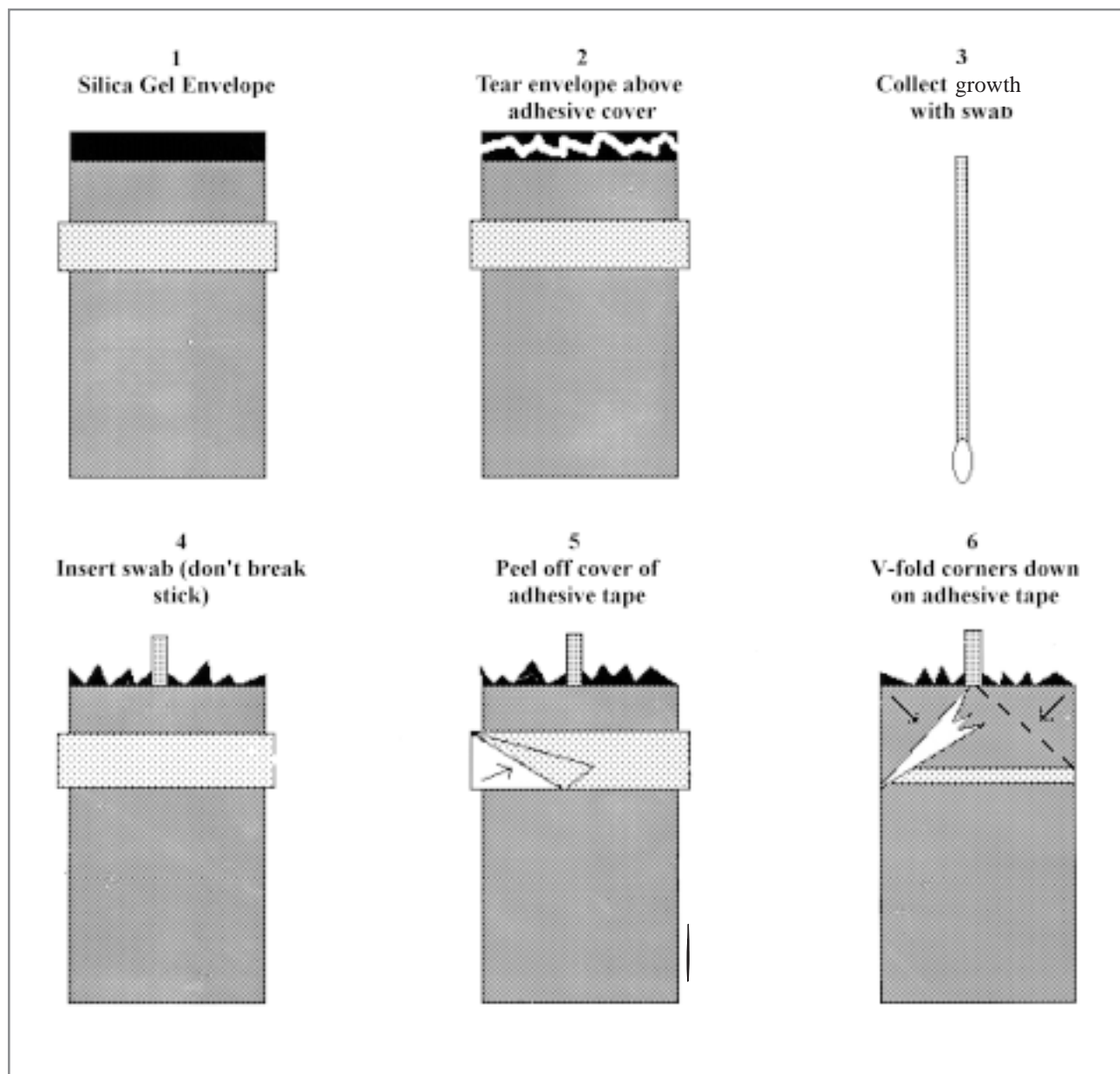


Figure 25. Instructions for using silica gel envelopes for transport of isolates.

- (a) Grow the bacteria on BAP or CAP (*H. influenzae*). Incubate the plates in a CO₂ or candle-jar for 18-20 hours at 35 °C. Inspect the plate for purity.
- (b) Harvest all of the growth from a plate with a sterile swab.
- (c) Cut open the silica gel package and place the swab in it. Fold the corners and seal the silica gel package with the tape. Label the silica gel package with the appropriate identification number.
- (d) When the swabs in silica gel packages are kept at 4 °C, *N. meningitidis* can survive at least 3 weeks, and *S. pneumoniae* and *H. influenzae* at least 2 weeks. At room temperature, *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* can survive 2 weeks.

IX. Bibliography

1. Ajello GW, Feeley JC, Hayes PS, Reingold AL, Bolan G, Broome CV, and Phillips CJ. Trans-Isolate medium: a new medium for primary culturing and transport of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. J. Clin. Microbiol., 1984, 20:55-58.
2. Austrian, R. The Quellung reaction, a neglected microbiologic technique. Mt. Sinai J. Med., 1976, 43:699-703.
3. Campos, JM. *Haemophilus*. In: Manual of Clinical Microbiology, 6th ed. PR Murray, EJ Baron, MA Tenover, FC Tenover, RH Tenover (eds.), 1995:556-565. American Society for Microbiology, Washington, DC, USA.
4. Facklam, RR, and JA Washington II. Streptococcus and related catalase negative gram-positive cocci. In: Manual of Clinical Microbiology, 5th ed. A Balows, WJ Hausler, KL Herrmann, HD Isenberg, and HJ Shadomy (eds.), 1991:238-257. American Society for Microbiology, Washington, DC, USA.
5. Isenberg, HD, ed. Clinical Microbiology Procedures Handbook 1992, Volume #1. American Society for Microbiology, Washington, DC.
6. Knapp, JS, and RJ Rice. *Neisseria and Branhamella*. In: Manual of Clinical Microbiology, 6th ed. PR Murray, EJ Baron, MA Pfaller, FC Tenover, RH Tenover (eds.), 1995: 324-340. American Society for Microbiology, Washington, DC, USA.
7. Miller, MJ. A guide to specimen management in clinical microbiology. Microbiology technical services, Dunwoody, GA, and Diagnostic Microbiology Section, Hospital Infections Program, Centers for Disease Control and Prevention, Atlanta, GA, USA, 1996.
8. Schwartz, B, and RR Facklam. Manual for the National Surveillance of Antimicrobial Resistance of *S. pneumoniae* and *H. influenzae*: Epidemiological and Microbiological Methods. World Health Organization, Geneva, Switzerland, and Centers for Disease Control and Prevention, Atlanta, GA, USA, 1994.
9. Sørensen, UBS. Typing of pneumococci by using 12 pooled antisera. J. Clin. Microbiol., 1993, 31:2097-2100.
10. Van der Ende, A, IGA Schuurman, CTP Hopman, CAP Fijen, and J Dankert. Comparison of commercial diagnostic tests for identification of serogroup antigens of *Neisseria meningitidis*. J. Clin. Microbiol., 1995, 33: 3326-7.
11. Control of Epidemic Meningococcal Disease: WHO Practical Guidelines, 2nd edition. World Health Organization, Geneva, Switzerland, 1998.
12. Murray CJL, Lopez AD. Global Health Statistics: a compendium of incidence, prevalence, and mortality estimates for over 200 conditions, 1996: 283-309. The Harvard School of Public Health on behalf of the World Health Organization and the World Bank, Boston, MA, USA.

X. Annexes

Annex A. Basic Requirements, Supplies and Equipment

A1. Table 3: Basic Requirements and Supplies for a Microbiology Laboratory

Basic Laboratory Requirements	Supplies	Equipment
<p>good lighting electricity gas supply for burners bench space (4-6 ft/person) distilled water supply desk lamps sink with running water</p>	<p>laboratory coats gloves disinfecting fluid (Amphyl) sharp disposal box (needles) containers for waste disposal labels marking pens record books towels hand soap inoculating loops scissors forceps candle jars 3 x hand lens spatulas or spoons rulers or calliper test tube racks required media thermometers lens paper lens cleaner immersion oil pH paper standard buffers autoclave tape Pasteur pipettes microscope slides</p>	<p>autoclave refrigerator incubator weight balance pH meter timers Bunsen or alcohol burners microscope with immersion lens water bath freezer safety hood</p>

**A2. Table 4: Equipment and Supplies Needed for Collection of Clinical Specimens
and Isolation and Identification of *N. meningitidis*, *S. pneumoniae*
and *H. influenzae***

Procedure	Supplies	Equipment
1. Spinal tap kit for preparation of patient and collection of CSF	adult spinal needle 22 gauge 3.5" paediatric spinal needle 23 gauge 1.5" or 2.5" alcohol spongettes gauze sponge 4x4" Band-Aids tincture of iodine or povidone-iodine swabs sterile screw-cap tubes syringe needle mailing containers T-I medium	
2. Preparation of patient and drawing of blood	iodine swab alcohol swab tourniquets syringes needles gloves bandages cotton gauze scissors puncture-resistant container blood culture media or transport system	
3. Inoculation of CSF and blood culture media	blood culture media needles aeration needles (vents) alcohol wipes	
4. Incubation of inoculated media	candles	candle-jar or CO ₂ incubator

5. Subculturing of inoculated media and blood culture bottles	candles alcohol wipes inoculating loops	Bunsen or alcohol burners candle-jar or CO ₂ incubator
6. Transport and culture of media	silica gel packages polyester-tipped swabs packing materials labels Styrofoam containers	
7. Sterilization of disposable and contaminated materials	containers for autoclave	autoclave
8. Identification	chromatography paper such as Whatman 3MM immersion oil inoculating loop inoculating needle pipettes pipette bulb capillary pipette pipette tips pipette man glass slides cotton swabs 0.5 McFarland turbidity standards forceps test tube racks scissors	microscope (oil immersion) magnifying glass or broad field microscope

<p>9. Permanent storage of strains</p>	<p>vials or ampoules</p>	<p>-70 °C or N₂ freezer -20 °C freezer for shorter storage lyophilizer</p>
<p>10. Media preparation</p>	<p>Petri dishes sterile cotton swabs test tubes Erlenmeyer flasks (250 ml, 500 ml, 1 l, 2 l) beakers (100 ml, 250 ml, 500 ml) weighing papers holders distilled water glass beads pipettes pipette bulbs spatulas sterilization filter units cylinders (50 ml, 100 ml, 250 ml, 500 ml, 1 l)</p>	<p>weighing balance refrigerator incubator magnetic stirrer autoclave pH meter timer</p>

Annex B. Media and Reagents

Note : The following list of the manufacturers of the commonly used media and reagents does not indicate special endorsement of these products and/or manufacturers by WHO. Please follow closely the manufacturer's instructions when using these media and reagents.

B1. Table 5: Media and Reagents Necessary for Isolation and Identification of *N. meningitidis*, *S. pneumoniae* and *H. influenzae**

Reagents/ Media	BD	CSM	Sigma	Difco	BBL	Oxoid	bioMérieux	Sanofi Pasteur	Unit
Blood agar base							51 431	64524	500 g
Trypticase soy agar	4311043			0369-17-6	11043	CM131B	51 461	64554	500 g
Trypticase soy agar ^v	4321283			2071-30-0	21283				100 plates
Trypticase soy agar with 5 % sheep blood ^v	4321239 4321261				21239 21261		43 001	63964	20 plates 100 plates
Mueller-Hinton 2 agar with sheep blood ^v	4321176				21176 21274		43 321	63825	20 plates 100 plates
Mueller-Hinton agar	4311438			0252-17-6	11438	CM337B		64884	500 g
Horse blood	11949				11949	SR50	55 832	56642	100 ml 50 ml
Sheep blood	11946				11946	SR49	55 822	56652	100 ml 50 ml
Trypticase soy broth	4311768		T8907	0370-17-3	11768	CM129B	51 141	64144	500 g
Trypticase soy broth with SPS & CO ₂ ^v				1712-37-4	21480				10 x 50 ml
Heart infusion broth	99532			0038-17-7					500 g
Heart infusion agar	12435			0044-17-9					500 g
Mueller-Hinton broth	11443			0757-17-6	11443	CM405B		69444	500 g
Reagents/ Media	BD	CSM	Sigma	Difco	BBL	Oxoid	bioMérieux	Sanofi Pasteur	Unit

Trypticase soy broth ^ψ	4321716				21716				100 x 5 ml
GC agar	4311275			0289-17-3	11275	CM367B			500 g
Chocolate agar plates ^ψ	4321169				21169		43 101	63934	20 plates 100 plates
Chocolate agar slants ^ψ	4321348				21348				10 slants
Haemoglobin, dried bovine	11871				11871	LP053B			100 g
Haemoglobin (2% sol) ^ψ	11874			3248-73-8	B11874				10 x 100 ml
IsoVitaleX or Vitox ^ψ	11876				11876	SR090A	55 652	55592	5 x 10 ml
Sodium polyanethole sulfonate			P2008						
Haematin, bovine			H3505						
Haematin, chloride			H2250						
Dextrose ^ψ		02-065-02			21634				100 plates
Lactose ^ψ		02-066-02							100 plates
Maltose ^ψ		02-067-02			21638				100 tubes
Sucrose ^ψ		02-068-02			21646				100 tubes

Reagents/ Media	BD	CSM	Sigma	Difco	BBL	Oxoid	bioMérieux	Sanofi Pasteur	Unit
Crystal violet			C0775						
Safranin			S8884						
Iodine			I3380						
Potassium iodine			P8256						
Crystal violet ^v	12293				12293		55 545		4 x 250 ml
Iodine ^v	12294				12294		55 546		4 x 250 ml
Acetone-alcohol ^v	12295				12295		55 547		4 x 250 ml
Safranin ^v	12296				12296		55 548		4 x 250 ml
Ethanol									
Acetone									
Barium chloride			B6394						
1 N sulfuric acid			84728						
1 N sodium hydroxide			930-65						
Sodium hydroxide pellets			S0899						
Dextrose	11863			0155-17-4		11863		64034 62242	500 g (30%) 5 ml
Maltose	12029			0168-17-9		12029		62361	500 g (30%) 5ml

Reagents/ Media	BD	CSM	Sigma	Difco	BBL	Oxoid	bioMérieux	Sanofi Pasteur	Unit
Lactose	11881			0156-17-3		11881		62272	500 g (25%) 10 ml
Sucrose	11916			0176-17-9		11916		62571	500 g (30%) 10 ml
1N hydrochloric acid			920-1						
Kovac's oxidase reagent			T3134						
V-factor disks ^v	31108			1694-90-0		DD004A			50 discs
X-factor disks ^v	31006			1695-90-9		DD003A			50 discs
XV-factor disks ^v	31104			1696-90-8		DD005A			50 discs
Haemophilus quad ID	4397890								
Optochin p-discs	31046			1632-35-3	31046	DD001A		53852	30-50 discs
Nicotinamide adenine dinucleotide			N7004						

Reagents/ Media	BD	CSM	Sigma	Difco	BBL	Oxoid	bioMérieux	Sanofi Pasteur	Unit
T-I medium -supplement B -activated charcoal -soluble starch -agar -trypticase soy broth -gelatin -3 LN- morpholino propanesulfonic acid (MOPS) bovine albumin	4311768		C5510 S9765 A5306 T8907 M5162 A9647	0276-60-4 0140-01-0 0370-17-3 0143-17-9		11768		64144	6 x 10 ml 1 lb 500 g 500 g

*BD - Becton Dickinson and Co., CSM - Carr-Scarborough Microbiologicals, Inc., Sigma - Sigma Chemical Co., Difco - Difco Laboratories, BBL - BBL Microbiology Systems. For the manufacturers' addresses and contact numbers, please see section B4.

ψ Although commercially prepared media and reagents are more expensive than media or reagents that can be prepared locally, there will be times when commercially available items will be preferable. It may be desirable to purchase the supply of media and reagents to perform short-term studies rather than attempt formulation. The media and reagents designated with (ψ) are available in ready-to-use form in most parts of the world. It is essential that each medium lot have a satisfactory expiry date and that this date and lot number be recorded in the laboratory, and noted for quality and control testing.

B2. Table 6: Commercially Available Tests for Latex Agglutination, Co-Agglutination and Serogrouping/Serotyping of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*

Latex Agglutination

Test	Suppliers*	Product Information
Directigen	Becton-Dickinson Microbiology Systems	Cat # 4950160: <i>N. meningitidis</i> groups A/Y and C/W135 Kit Cat # 4952260: <i>H influenzae</i> type b Kit Cat # 4951960: <i>S. pneumoniae</i> Kit Cat # 4952360: Meningitis Combo Test Kit for <i>N. meningitidis</i> groups C/W135, A/Y, B/E. <i>coli</i> K1, <i>H. influenzae</i> type b, <i>S. pneumoniae</i> , and group B streptococcus
MicroScan	VWR Scientific	Cat # B1049-6 ImmunoSCAN Direct: <i>S. pneumoniae</i> Latex Test Kit Cat # B1049-7 ImmunoSCAN Direct: Meningitis Test Kit for <i>N. meningitidis</i> groups A/C, Y/W135
Pastorex	Sanofi Diagnostics Pasteur	Cat # 61701: Pastorex Meningitis Kit for <i>N. meningitidis</i> groups A, B, C, Y, W135, <i>H. influenzae</i> type b, <i>S. pneumoniae</i> and group B streptococcus
Slidex	bioMérieux	Cat # 58 803: Slidex méningite Kit 5 for detection of <i>N. meningitidis</i> groups A, C, B/ <i>E. coli</i> K1, <i>H. influenzae</i> type b, and <i>S. pneumoniae</i>
Wellcogen	Murex Diagnostics, Inc.	Cat # ZL26: Wellcogen Bacterial Antigen Kit for detection of <i>N. meningitidis</i> groups A/C/Y/W135, B/E. <i>coli</i> K1, <i>H. influenzae</i> type b, <i>S. pneumoniae</i> Cat # ZL21: Wellcogen <i>H. influenzae</i> type b and group B streptococcus Cat # ZL22: Wellcogen <i>S. pneumoniae</i> Cat # ZL23: Wellcogen <i>N. meningitidis</i> groups A/C/Y/W135 Cat # ZL24: Wellcogen <i>N. meningitidis</i> B/E. <i>coli</i> K1

*For the manufacturers' addresses and contact numbers, please see section B4.

Co-Agglutination

Test	Supplier*	Product Information
Phadebact	Pharmacia Diagnostics (US distributor REMEL)	Cat # 60-401: CSF test for <i>N. meningitidis</i> groups A/B/C/Y/W135, <i>H. influenzae</i> type b, <i>S. pneumoniae</i> , and group B streptococcus; CSF pneumococcus test; CSF Haemophilus test

*For the manufacturers' addresses and contact numbers, please see section B4.

Serogrouping/Serotyping Antisera

Test	Supplier*	Product Information
Serogrouping antisera	Murex Diagnostics, Inc.	Cat # ZM 33: Polyvalent set 1 of <i>N. meningitidis</i> agglutinating antisera Cat # ZM 34: Polyvalent set 2 of <i>N. meningitidis</i> agglutinating antisera Cat # ZM 37: <i>N. meningitidis</i> antiserum group A Cat # ZM 51: <i>N. meningitidis</i> antiserum group B Cat # ZM 39: <i>N. meningitidis</i> antiserum group C Cat # ZM 44: <i>N. meningitidis</i> antiserum group W135 Cat # ZM 41: <i>N. meningitidis</i> antiserum group X Cat # ZM 42: <i>N. meningitidis</i> antiserum group Y Cat # ZM 43: <i>N. meningitidis</i> antiserum group Z
Serogrouping antisera	Difco Laboratories	Cat # 2228-50-1: <i>N. meningitidis</i> antiserum group A Cat # 2229-50-0: <i>N. meningitidis</i> antiserum group B Cat # 2230-50-7: <i>N. meningitidis</i> antiserum group C Cat # 2253-50-9: <i>N. meningitidis</i> antiserum group W135 Cat # 2880-50-0: <i>N. meningitidis</i> antiserum group X Cat # 2281-50-9: <i>N. meningitidis</i> antiserum group Y Cat # 2891-50-7: <i>N. meningitidis</i> antiserum group Z Cat # 2252-50-5: <i>N. meningitidis</i> antiserum group Z' Cat # 2232-50-5: Antiserum Poly - polyvalent antiserum for <i>N. meningitidis</i> A/B/C/D Cat # 2910-50-4: Antiserum Poly 2 - polyvalent antiserum for <i>N. meningitidis</i> X/Y/Z

Slidex pneumo-kit	bio-Mérieux	Cat # 58821: <i>S. pneumoniae</i>
Pneumoslides	Becton-Dickinson Microbiology Systems	Cat # 40840: <i>S. pneumoniae</i>
Serotyping antisera	Difco Laboratories	Cat # 2237-50-0: polyvalent <i>H. influenzae</i> antisera Cat # 2250-50-2: <i>H. influenzae</i> antisera type a Cat # 2236-50-1: <i>H. influenzae</i> antisera type b Cat # 2789-50-2: <i>H. influenzae</i> antisera type c Cat # 2790-50-9: <i>H. influenzae</i> antisera type d Cat # 2791-50-8: <i>H. influenzae</i> antisera type e Cat # 2792-50-7: <i>H. influenzae</i> antisera type f
Serotyping antisera	Murex Diagnostics, Inc.	Cat # ZM 20: <i>H. influenzae</i> antisera type a Cat # ZM 21: <i>H. influenzae</i> antisera type b Cat # ZM 22: <i>H. influenzae</i> antisera type c Cat # ZM 23: <i>H. influenzae</i> antisera type d Cat # ZM 24: <i>H. influenzae</i> antisera type e Cat # ZM 25: <i>H. influenzae</i> antisera type f

*For the manufacturers' addresses and contact numbers, please see section B4.

Note: Slashes (/) indicate polyvalent diagnostic reagents and commas (,) indicate monovalent diagnostic reagents.

B3. Table 7: Control Strains for Quality Assurance Testing of Media and Reagents

Medium	Control Organism	ATCC No.	Expected Results
Blood agar base	<i>Streptococcus pneumoniae</i> <i>Streptococcus pyogenes</i> <i>Staphylococcus aureus</i> <i>Escherichia coli</i>	6035 19615 25923 25922	Growth, α haemolysis Growth, β haemolysis Growth Growth
Tryptic soy agar with sheep blood	<i>Streptococcus pneumoniae</i> <i>Streptococcus pyogenes</i> <i>Staphylococcus aureus</i> <i>Escherichia coli</i>	6305 19615 25923 25922	Growth, α haemolysis Growth, β haemolysis Growth Growth
Tryptic soy (aerobic) Plain agar or broth	<i>Streptococcus pyogenes</i> <i>Escherichia coli</i>	19615 25922	Growth Growth
Blood culture-aerobic bottle	<i>Pseudomonas aeruginosa</i> <i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i>	27853 6305 10211	Growth Growth Growth
Chocolate agar	<i>Neisseria gonorrhoeae</i> <i>Haemophilus influenzae</i>	43069 10211	Growth Growth
Glucose	<i>Neisseria gonorrhoeae</i> <i>Branhamella catarrhalis</i>	43069 25240	Yellow No colour or deep red
Lactose	<i>Neisseria lactamica</i> <i>Neisseria gonorrhoeae</i>	23971 43069	Yellow No colour or deep red
Maltose	<i>Neisseria meningitidis</i> <i>Neisseria gonorrhoeae</i>	13090 43069	Yellow No colour or deep red
Sucrose	<i>Neisseria sicca</i> <i>Neisseria gonorrhoeae</i>	9913 43069	Yellow No colour or deep red
Oxidase test	<i>Pseudomonas aeruginosa</i> <i>Escherichia coli</i>	27853 25922	Purple No colour
Optochin test and bile solubility test	<i>Streptococcus pneumoniae</i>	49619	Optochin susceptible Bile soluble
X and V factor requirements	<i>Haemophilus influenzae</i>	49247	Requires both X and V for growth

ATCC: American Type Culture Collection.

B4. Manufacturers' Addresses:

Becton-Dickinson

250 Schilling Circle
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Annex C. Preparation of Media and Reagents

C1. Quality Control of All Media

Immediately after preparation, each medium should be tested with a reference strain of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* for proper growth characteristics as described for each medium. Keep a record of all media preparation or purchase dates and quality control test results. Note any unusual characteristic such as colour of the medium or slow growth of test bacteria.

C2. Routine Agar and Broth Media

All agar media can be dispensed into 15x100-mm Petri dishes (15 to 20 ml per dish). After pouring, leave the plates at room temperature for several hours to prevent excess condensation from forming on the covers of the dishes. Place the plates and store them in a plastic bag in an inverted position at 4 °C.

Heart Infusion Agar (HIA) and Trypticase Soy Agar (TSA)

These media are general purpose media used with or without blood for isolating and cultivating a number of microorganisms. The media should appear straw colored (a yellowish to gold colouring). HIA and TSA are also used for determining the X- and V-factor requirements of *H. influenzae*. Each freshly prepared or purchased batch of HIA or TSA should be quality control tested by determining the X and V requirements of *H. influenzae*. A fresh plate is inoculated with a control strain; X, V, and XV disks should be placed on the inoculated plate identical to that shown in Figure 23. *H. influenzae* should grow only around the XV disk.

- (a) Prepare the HIA or TSA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks. These media should be fully dissolved with no powder on the walls of the vessel, before autoclaving. Autoclave at 121 °C for 20 minutes.
- (b) Cool to 50 °C and pour into 15x100-mm Petri dishes.
- (c) Allow to solidify and condensation to dry out before placing in plastic bags and storing at 4 °C until used.

Blood Agar Plate (BAP): TSA + 5% Sheep Blood

BAP is used as a general blood agar medium (sheep blood agar plate). It is also used for the optochin test and subcultures of *S. pneumoniae*. The plate should appear a bright red colour. If the plates appear dark red, the blood has been added when the agar was too hot. If this happens, discard the medium and prepare another batch. Test each new, freshly prepared or purchased batch of BAP for growth and haemolytic reaction with a strain of *S. pneumoniae*. The colonies are small and should appear grey to grey-green surrounded by a distinct greenish halo in the agar similar to that shown in Figure 12.

- (a) Prepare TSA according to the instructions given on the label of the dehydrated powder. It is convenient to prepare 500 ml of molten agar in a 1-litre flask. Add 20 g of agar into 500 ml of water. Heat to dissolve.
- (b) Autoclave at 121 °C for 20 minutes. Cool to 60 °C.
- (c) Add 5% sterile, defibrinated sheep blood (25 ml sheep blood is added to 500 ml of agar). If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (e.g. 50 ml of blood per litre of medium).
- (d) Dispense 20 ml into 15x100-mm Petri dishes. Allow to solidify, dry out, place in a plastic bag, and store at 4 °C.

Heart Infusion Rabbit Blood Agar Plate (HIA - Rabbit Blood)

HIA-rabbit blood is used for determining the haemolytic reaction of *Haemophilus* species. This medium should appear bright red and look very similar to BAP. If the medium is dark red, discard and prepare a new batch. Horse blood may be substituted for rabbit blood in this medium. A strain of *H. haemolyticus* should be used to quality control the proper growth and haemolytic reactions of the medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete haemolysis which appears as a clear halo surrounding the colonies.

- (a) Prepare the HIA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks and autoclave at 121 °C for 20 minutes. Cool to 50 °C in a water bath.
- (b) Add 5% sterile, defibrinated rabbit blood (5 ml/100 ml of medium) and dispense into 15x100-mm Petri dishes. Allow to solidify and dry for a few hours. Then, place in a plastic bag and store at 4 °C.

Horse Blood Agar (Blood Agar Base)

This highly nutritive medium may be used for the primary isolation of *H. influenzae* and for the determination of the haemolysis with *H. haemolyticus* or other bacteria. The quality control testing of this medium should be the same as that described for HIA-rabbit blood.

- (a) Prepare blood agar base according to the instructions on the label of the dehydrated medium. Oxoid number 2 base is best, but other blood agar bases may be substituted.
- (b) Autoclave at 121 °C for 15 minutes, and cool to 50 °C in a water bath.
- (c) Add horse blood (5 ml/100 ml of medium).
- (d) Mix well, dispense in 15x100-mm Petri dishes. Allow to solidify and dry out excess moisture before placing in plastic bags and storing at 4 °C.

Trypticase Soy Broth (TSB)

TSB is used for making suspensions of *H. influenzae* prior to testing for X- and V-factor requirements. Heart infusion broth, sterile saline or PBS may be substituted for TSB. *H. influenzae* does not grow in TSB but the medium should not be toxic to other bacteria. Therefore, *S. pneumoniae* should be used to quality control for toxicity. Inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae*;

incubate overnight at 35 °C. The broth should be turbid the next day. Subculture the broth to test for proper growth characteristics of *S. pneumoniae* (use a BAP).

- (a) Prepare the TSB according to the instructions on the label of the dehydrated medium.
- (b) Dispense 5 ml into 15x125-mm tubes, autoclave at 121 °C for 20 minutes, cool, and store at 4 °C.

Blood Culture Broths

Each new batch of freshly prepared or purchased blood culture medium should be tested for supporting the growth of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. Add 1 to 3 ml of sterile rabbit, horse, or human blood to three bottles of freshly prepared blood culture medium. A fresh culture of each of the three bacteria should be inoculated into separate blood culture bottles. Make a dilute suspension of growth from an agar plate in broth by collecting a loopful of the growth and suspending it in 1-2 ml of broth. Inoculate this suspension into the blood culture broth to be tested. Incubate the broths at 35 °C for 7 days, observe for growth and subculture at 14 hours and 48 hours. All three bacteria should be recovered on subculture after 14 and 48 hours.

- (a) Follow the instructions of the manufacturer on the label of each bottle of dehydrated TSB.
- (b) Add 0.25 g SPS per litre of medium. SPS is especially important for recovery of *H. influenzae*.
- (c) Dispense in 20-ml (paediatric blood culture bottle) and 50-ml (adult blood culture bottle) amounts into suitable containers (tubes or bottles) with screw-caps with rubber diaphragms. The amount of liquid in the containers should make up at least two-thirds of the total volume of the container.
- (d) Sterilize by autoclaving at 121 °C for 15 minutes. Cool and store medium at room temperature.

C3. Special Media

Chocolate Agar Plate (CAP)

CAP with growth supplements is a medium that supports the special growth requirements needed for the isolation of fastidious organisms, such as *H. influenzae* and *N. meningitidis* when incubated in a 5% CO₂ atmosphere. CAP has reduced concentration of agar, which increases the moisture content of the medium.

Supplemented chocolate agar should support the growth of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*. Chocolate agar slants for transport and short-term storage can be prepared in the same manner as that described for agar plates, except that the medium is dispensed in 16x125-mm screw-cap tubes and slanted before solidifying. All freshly prepared or purchased chocolate agar media should be tested with the three species of bacteria to determine the medium's capacity to support their growth. Chocolate agar slants should look brown to brownish-red in colour. *N. meningitidis* and *H. influenzae* should appear as a greyish, almost translucent film on the slant's surface with

no discolouring of the medium after 24 hours of incubation. *S. pneumoniae* should appear as small grey to grey-green colonies with a very distinct greenish discolouring of the medium (see Figures 7 and 12 for these reactions). If the medium does not support the growth of one or all of the bacteria, discard and prepare or purchase a fresh batch. If *H. influenzae* does not grow, the IsoVitaleX or its equivalent may have been inadvertently omitted.

Chocolate Agar with TSA and Growth Supplement

- (a) Use TSA as the basal medium. Prepare double strength (20 g in 250 ml distilled water). Autoclave, and cool to 50 °C. Use the thermometer to verify the cooling temperature.
- (b) Haemoglobin solution: Prepare a solution of 2% haemoglobin (5 g in 250 ml distilled water). Mix the haemoglobin in 5-6 ml of the distilled water to form a smooth paste. Continue mixing as the rest of the water is added. Autoclave, and cool to 50 °C.
- (c) Add the haemoglobin solution to the double-strength TSA and continue to hold at 50 °C.

Alternative to steps a-c: If a haemoglobin solution is unavailable, an alternative is to add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood per 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water). DO NOT use human blood. After the base medium has been autoclaved and cooled to 50 °C, add the blood and place in a hot water bath at no more than 80 °C for 15 minutes or until a chocolate colour is achieved. Then cool to 50 °C.

- (d) Supplement: after the haemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50 °C, add growth supplement (such as IsoVitaleX or Vitox) to a final concentration of 1%. Mix the ingredients by gently swirling the flask; avoid forming bubbles.
- (e) Dispense 15 to 20 ml in each 15x100-mm Petri dish.

Chocolate Agar with Gonococcus Medium (GC) Base and Growth Supplement

- (a) Suspend 7.2 g of GC agar base in 100 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
- (b) Autoclave the bottle at 121 °C for 15 minutes. Cool to 50 °C.
- (c) Add 100 ml of warm distilled water to 2 g of soluble haemoglobin powder. Mix the powder with 5-10 ml of water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water. Autoclave the solution at 121 °C for 15 minutes. Cool to 50 °C. Alternatively, use 100 ml ready-made 2% sterile haemoglobin solution, warmed to 50 °C.
- (d) Reconstitute the growth supplement (e.g. IsoVitaleX or Vitox): reconstitute each lyophilized vial by aseptically transferring with a sterile needle and syringe 10 ml of the accompanying diluent. Shake to assure complete solution. After reconstitution, use immediately, or store at 4 °C and use within 2 weeks.
- (e) Aseptically add 100 ml sterile haemoglobin solution and growth supplement to 100 ml of GC agar base solution (2 ml of IsoVitaleX or 4 ml of Vitox). Mix gently, but thoroughly, to avoid air bubbles in agar.
- (f) Dispense 15 to 20 ml into sterile 15x100-mm Petri dishes.

- (g) Allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4 °C.

Cystine Trypticase Agar (CTA) with 1% Carbohydrate (a semi-solid medium)

- (a) Suspend 28 g of CTA medium (BBL) or 29.5 g (Difco) in 900 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
- (b) Autoclave the flask at 121 °C for 15 minutes. Cool to 50 °C.
- (c) Prepare 10% glucose solution using 10 g glucose in 100 ml of distilled water. Filter sterilize using a 0.22 micron filter.
- (d) Aseptically add this entire solution (100 ml of 10% glucose solution) to the 900 ml of the CTA medium to obtain a final 1% concentration of the glucose.
- (e) Dispense 7 ml in 16 x 125 mm glass tubes with screw caps.
- (f) Store at 4 °C.
- (g) Repeat this procedure for the remaining 3 carbohydrates: maltose, lactose and sucrose.

Levinthal's Medium for Haemophilus spp.

Solution A: Levinthal's stock solution

- (a) Suspend 37 g of brain heart infusion broth base into 450 ml of endotoxin-free water. Mix thoroughly and heat to boiling to dissolve powder.
- (b) Add 50 ml of defibrinated rabbit blood to the hot mixture and mix well.
- (c) Cool and filter through gauze or glass wool.
- (d) Centrifuge the liquid portion at 12,000 revolutions per minute for 50 minutes.
- (e) Filter sterilize, and dispense in 100-ml amounts.

Solution B: agar

- (a) Add 7.5 g of agar to 500 ml of endotoxin-free water. Heat to dissolve.
- (b) Autoclave at 121 °C for 20 minutes.

The final agar medium is prepared by adding 500 ml of filter sterilized Levinthal's solution to 500 ml of sterile agar base at 55 °C. Aseptically dispense into sterile Petri dishes.

C4. Transport and Storage Media

Trans-Isolate (T-I) Medium

T-I medium is a biphasic medium that is useful for the primary culture of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* from CSF and blood samples. It can be used as a growth medium as well as a holding and transport medium.

- (a) Use glass serum bottles with flange-type, slotted rubber plugs and aluminum crimp seals. Any size from 20- or 30-ml capacity or greater will do, provided that the combined volume of solid and liquid phases equals approximately one-half the capacity of the bottle.

Diluent for solid and liquid phases:

- a 3- (N-morpholino) propanesulfonic acid (MOPS) buffer
0.1 M, pH 7.2 20.93 g
- b Distilled water up to 1000 ml after adjusting to pH 7.2 with NaOH

(b) Solid phase:

- Activated charcoal 2.0 g
- Soluble starch 2.5 g
- Agar (Difco) 10.0 g

Suspend in 500 ml of MOPS buffer and add a magnetic bar to the flask. Heat on a magnetic stirrer-heater to dissolve the starch and the agar. While stirring to keep the charcoal in suspension, dispense 5.0 ml to each 20-ml serum bottle. Cap each bottle with a piece of aluminium foil and autoclave in metal baskets at 121 °C for 20 minutes. Remove from the autoclave and slant the baskets until the bottles cool, so that the apex of the agar reaches the shoulder of each bottle.

(c) Liquid phase:

- Tryptic soy broth (Difco) 30.0 g
- Gelatin (Difco) 10.0 g
- MOPS buffer 500.0 ml

Heat the medium to dissolve the gelatin and avoid coagulation. Then autoclave at 121 °C for 15 minutes. To permit use of the medium for blood culture, add aseptically when cool:

- SPS 250.0 mg

Dissolve by mixing vigorously in 5 ml or less of MOPS buffer.

Pass through a 0.22- μ membrane filter to sterilize before adding to the medium.

Optional additive: Ten ml of the growth supplement (Vitox or IsoVitaleX) to help support growth of *H. influenzae* can be added aseptically to cooled medium. Alternatively, add 0.1 ml of the supplement to an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.

(d) Dispense 5 ml of the broth aseptically into each of the bottles containing the solid-phase slants. Seal with sterile rubber stoppers and aluminum caps. Use a hand-crimping tool to fasten the aluminium caps if an automated system is not available. T-I bottles can be stored and used for at least 2 years if tightly capped and stored at 4 °C. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. Before use, check several inoculated bottles for their ability to support meningococcal growth as well as some uninoculated bottles for sterility at 35 °C. Before inoculation, the bottles should be pre-warmed in the incubator (35 °C-37 °C) or allowed to reach room temperature (25 °C-30 °C).

Greaves solution for preservation of strains by freezing

- Albumin, bovine, fraction V 10.0 g

L-glutamic acid, sodium salt (Fluka, Buchs, Switzerland, 49621)	10.0 g
Glycerol	20.0 ml
Distilled water	200.0 ml

Mix all ingredients and let them dissolve for 2-3 hours. Sterile filter the solution. Transfer the solution to a sterile tube. Incubate for 2 days at 35 °C-37 °C to control the sterility of the medium. Store at 4 °C.

C5. Miscellaneous Reagents

Reagents for Gram Stain (Hucker Modification)

(a) Ammonium oxalate-crystal violet

Solution a.

Crystal violet (certified)	2.0 g
Dissolve in 95% ethyl alcohol	20.0 ml

Solution b.

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Mix solutions a. and b. Let stand overnight. Filter through coarse filter paper before use.

(b) Gram's iodine (**protect solution from light**)

Iodine (crystalline)	1.0 g
Potassium	2.0 g
Distilled water	300.0 ml

Grinding the dry chemicals in a mortar with small additions of distilled water may be helpful in preparing the solution.

(c) Decolorizer: 95% ethyl alcohol

(d) Counterstain

a. Safranin

Stock solution:

Safranin-O (certified)	2.5 g
95% ethyl alcohol	100.0 ml

Working solution:

Safranin stock solution	10.0 ml
Distilled water	90.0 ml

b. Ziehl-Nielsen carbol-fuchsin (considered by many to be a more effective counter-stain than safranin)

Basic fuchsin	0.3 g
95% ethyl alcohol	10.0 ml
Phenol crystals, melted	5.0 ml
Distilled water	95.0 ml

Dissolve fuchsin in alcohol. Add the 5% phenol solution. Let stand overnight. Filter through coarse filter paper.

Note: This solution can be used as described or diluted 1:10. Alternatively, counterstain can be prepared as a 0.3-0.5 aqueous solution of the basic fuchsin.

Gram-stain kits or individual reagents are available commercially from several laboratory supply companies.

McFarland Turbidity Standards

Prepare a 1% solution of anhydrous BaCl_2 (barium chloride) and a 1% solution of H_2SO_4 (sulfuric acid). The McFarland standards consist of mixtures of the two solutions in various proportions to form a turbid suspension of BaSO_4 . A 0.5 McFarland standard is prepared by mixing 0.05 ml of the 1% barium chloride solution with 9.95 ml of 1% sulfuric acid solution, while 1.0 McFarland standard is prepared by mixing 0.1 ml of the 1% barium chloride solution with 9.9 ml of 1% sulfuric acid solution. The resulting mixture is placed in a tube identical to that used for preparing the dilution used for the bile solubility test and the *Haemophilus* ID plate. The same size tube (screw-capped) and volume of liquid must be used. Store in the dark at room temperature when not in use. Prepare a fresh standard solution every 6 months. McFarland standard density solution will precipitate and clump over time, and it needs vigorous vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred. If it has, prepare a fresh standard.

Skin Antiseptic

Iodine tincture: add 100 ml of 70% isopropyl alcohol to 1 g of iodine.

Annex D. Serotyping of *S. pneumoniae*

Typing of the pneumococci isolated from patients with various clinical syndromes, such as sporadic cases of meningitis, is not usually necessary. However, in some studies, where the study protocols focus on evaluation of vaccine efficacy and transmission of organisms, it will be necessary to serogroup and serotype the pneumococci. The checkerboard typing system will sufficiently identify the serotypes of pneumococci in most cases. There will be studies that will require complete testing for all pneumococcal types and the isolates will have to be sent to a reference laboratory for identification of all 90 serotypes. The availability of Omniserum (Statens Seruminstitut, Copenhagen, Denmark), a pooled pneumococcal serum that reacts with all types, provides clinical microbiology laboratories with an invaluable reagent for rapid identification of pneumococci.

D1. Quellung Typing of *S. pneumoniae*

A Quellung reaction results when a type-specific antibody is bound to the pneumococcal capsular polysaccharide and causes a change in the refractive index of the capsule so that it appears “swollen” and more visible. The pneumococcal cell stains dark blue and is surrounded by a sharply demarcated halo, which represents the outer edge of the capsule (Figure 26). The light transmitted through the capsule appears brighter than either the pneumococcal cell or the background. Single cells, pairs, chains, and even clumps of cells may have Quellung reactions.

The Quellung reaction is traditionally used for the typing of pneumococci. This is the method of choice because it is easy, fast, accurate, and economical. Most laboratories do not type pneumococcal isolates because of the large number of diagnostic antisera required for typing; a total of 90 different pneumococcal types have been described. Types that exhibit close serological cross-reactivity are grouped together. Of the 90 types, 58 belong to 20 groups containing from 2 to 4 types (Table 8); a total of 46 different pneumococcal types or groups are currently known. Monovalent factor sera (rendered specific by multiple absorptions or by induction of immunological tolerance to cross-reacting types previous to immunization) for identification of types within groups will not be discussed in this manual.

In most parts of the world, about 90% of all pneumococcal strains isolated from blood or CSF belong to one of the 23 different types or groups represented in the 23-valent pneumococcal vaccine. A total of 7 pooled sera in addition to 21 type or group sera are needed in order to type or group these strains by the use of the traditional pneumococcal diagnostic antisera. However, this manual describes a simple checkerboard typing system, based on 12 pooled sera, intended for typing and/or grouping most pneumococci isolated from CSF or blood.

Antigen Preparation and Typing

The type and condition of a culture that is received in the laboratory will determine the procedure used to prepare a suitable cell suspension for observation of the Quellung reaction.

Performance of the Test

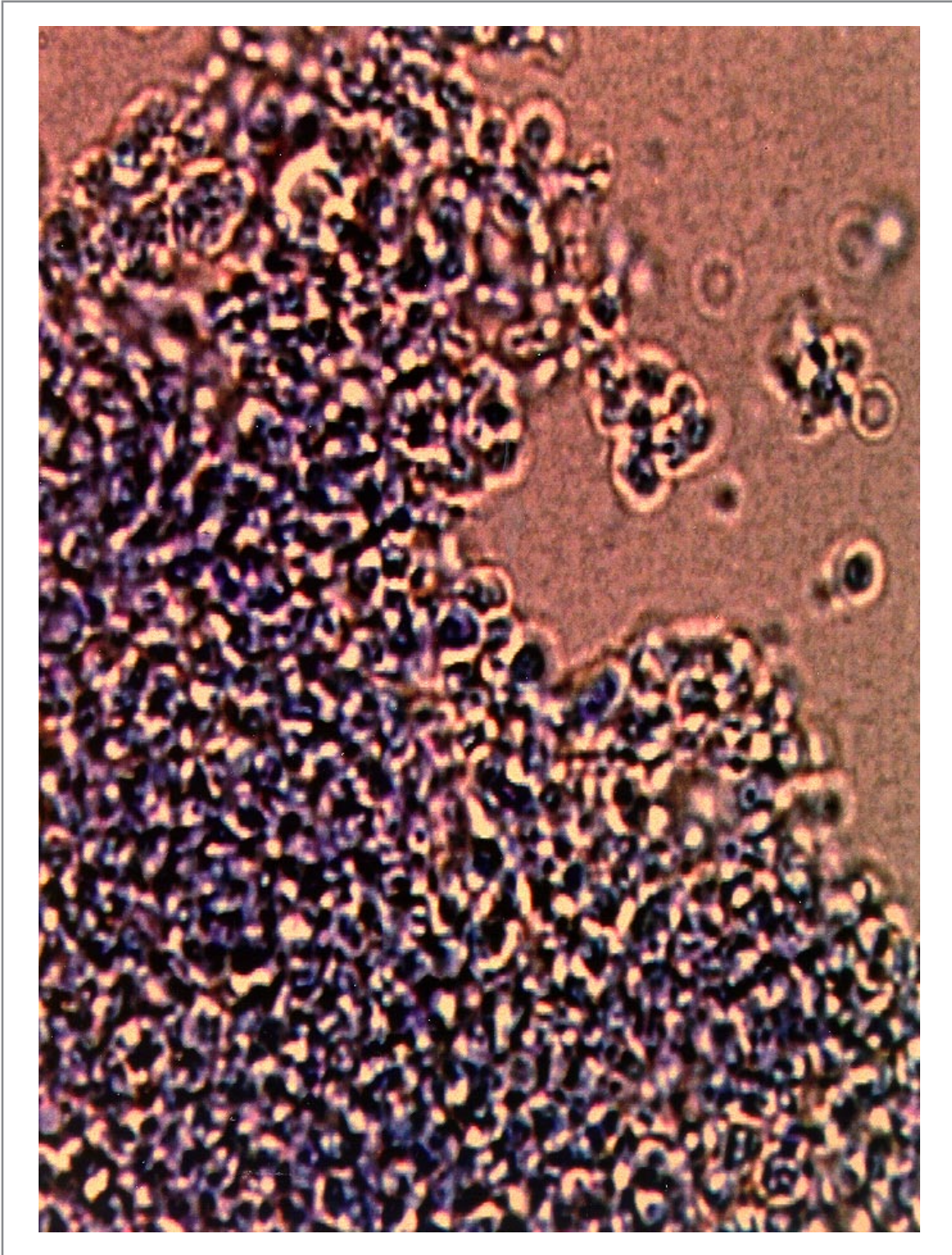


Figure 26. Quellung reaction for identification of *S. pneumoniae*.

- (a) Inoculate a BAP with an inoculating loop (always using a freshly prepared BAP). Inoculate about one-third of the plate heavily and streak the remainder of the plate for isolated colonies. Place the agar plate in a candle-jar or a carbon dioxide incubator for 18-24 hours at 35 °C.
- (b) Using a sterile loop, sweep across the surface of the 18-24 hours plate for inoculum and prepare a cell suspension equal to a 0.5 McFarland density standard in 0.5 ml of saline (0.85% sodium chloride). Optimum Quellung can be observed when there are 25 to 50 cells in a microscopic field.
- (c) With a loop or a micropipette, dispense equal amounts (5 to 10 µl) of the cell suspension and pneumococcal pool antiserum on a microscope slide. Add a small amount (1 loop) of 0.3% aqueous methylene blue and mix.
- (d) Cover the mixture with a 22-mm² coverslip and incubate at room temperature for 30 minutes.

Reading the Test Results

All positive Quellung reactions are shown in Figure 26. Note the clear area around the dark cell and the area outside the clear area (the capsule) in the dark background.

Non-Reactive Strains

- (a) If a Quellung reaction is not observed in one of the pools with the cell suspension from an agar plate, inoculate a 1.0 ml Todd-Hewitt broth, which has been supplemented with 2-3 drops of defibrinated sheep blood.
- (b) Incubate at 35 °C for 1-3 hours or until the broth above the blood is turbid.
- (c) Use one or two loops of the broth culture and test as in steps (c)-(d) above.
- (d) If a Quellung reaction is not observed in any of the pools, the identification of the strain as *S. pneumoniae* should be confirmed by re-testing for optochin susceptibility and bile solubility.

D2. Typing and/or Grouping of Pneumococci

The capsular reaction test is first carried out with, successively, the nine traditional pools (A to I) until a positive reaction is observed. Ordinarily, typing then proceeds by testing the strain in question with antisera against those individual types or groups that are included in the serum pool that gave a positive reaction. However, the checkerboard method described here proceeds by testing for a positive reaction with the serum pools (P to T). The type or group is then established from the reaction pattern by the use of a table with the types and groups entered in a rectangular checkerboard arrangement (Table 8).

Table 8: Checkerboard System for Typing and/or Grouping of Most Pneumococci Isolated From Blood or CSF^{a,b} (From Reference #9)

Existing Pool	Type or Group with New Pool					Non-Vaccine Related Type or Group
	P	Q	R	S	T	
A	1	18*	4	5	2	
B	19*	6*	3	8		
C					20	24*, 31, 40
D			9*		11*	16*, 36, 37
E			12*	10*	33*	21, 39
F				17*	22*	27, 32*, 41*
G						13, 28*
H ^c	14	23*		15*		29, 34, 35*, 42, 47*
I ^c						25, 38, 43, 44, 45, 46, 48

^a The five pooled sera P to T are composed in such a way that each of the 21 vaccine-related types and/or groups reacts both with one of these sera and with one of the seven pooled sera A to F plus H.

^b All 46 types of groups are shown in the table (Nos. 26 and 30 not being in use). Asterisks indicate groups containing the following types: **6**, 6A and **6B**; **7**, **7F**, 7A, 7B, and 7C; **9**, 9A, 9L, **9N**, and **9V**; **10**, 10F and **10A**; 11, 11F, **11A**, 11B and 11C; **12**, **12F** and 12A; **15**, 15F, 15A, **15B**, and 15C; 16, 16F and 16A; **17**, **17F** and 17A; **18**, 18F, 18A, 18B, and **18C**; **19**, **19F** and **19A**, 19B, and 19C; **22**, **22F** and 22A; **23**, **23F**, 23A, and 23B; 24, 24A, and 24B; 28, 28F and 28A; 32, 32F and 32A; **33**, **33F**, 33A, 33B, and 33C; 35, 35A, 35B, and 35C; 41, 41F and 41A; 47 and 47A. Types and/or groups present in the currently available 23-valent pneumococcal vaccine are indicated by boldface type.

^c Pools G and I do not react with vaccine types and are, therefore, not included in the chessboard system.

Annex E. Biotyping of *H. influenzae*

In the absence of Hib vaccination, *H. influenzae* isolated from the CSF of a patient with clinical meningitis will usually be Hib. Most laboratories will conclude their identification at that stage. However, in certain cases, reference laboratories may want to carry out the biotyping of *H. influenzae*. Biotyping can be performed irrespective of whether or not a serotype antigen was detected. The biotype is determined by the testing for indole, ornithine decarboxylase, and urease.

- (a) *Spot indole test*: Transfer a heavy inoculum (a 25- μ l loopful) to a piece of filter paper so that the bacteria are contained in a heavy spot. Add one drop of indole reagent (Kovac's reagent) to the cells. A blue colour will appear within 1 minute when the bacteria are producing indole. A red, pink or pinkish-yellow reaction is negative.
Do not read the test after 5 minutes, as all reactions may appear positive.
- (b) *Ornithine decarboxylase test*: Inoculate a tube of ornithine medium with a loopful of bacterial suspension (0.5 McFairland). This suspension can also be used to inoculate the *Haemophilus* ID medium described above. After inoculating the ID medium, cover the ornithine decarboxylase test tube media with sterile mineral oil. Incubate 4 hours or overnight at 35 °C. A purple colour is positive for the presence of the ornithine decarboxylase enzyme, and a yellow colour or no colour is negative.
- (c) *Urease test*: Inoculate a tube of Christensen's medium with a loopful of bacteria. Incubate 4 hours or overnight at 35 °C. A pink to red colour is positive, and no change or a yellow colour is negative.
- (d) The biotype is determined by matching the observed reactions to those described in Table 9.

Note: When performing these tests follow the specific instructions provided by the manufacturers as different colour indicators may be employed by different manufacturers.

Table 9: Identification of *H. influenzae* Biotypes

Test	Biotype							
	I	II	III	IV	V	VI	VII	VIII
Urease	+	+	+	+	-	-	-	-
Indole	+	+	-	-	+	-	+	-
Ornithine	+	-	-	+	+	+	-	-

XI. List of Figures

1. Kit for the collection of CSF.
2. Collection of CSF by lumbar puncture.
 - A. The patient lies on his side with knee flexed and back arched to separate the lumbar vertebrae. The patient is surgically draped, and an area overlaying the lumbar spine is disinfected.
 - B. The space between lumbar vertebrae L3 and L4 is palpated with the sterilely gloved forefinger.
 - C. The spinal needle is carefully directed between the spinous processes, through the intraspinal ligaments into the spinal canal.
3. Collection of blood from an arm.
 1. Apply the tourniquet.
 2. Select a vein.
 3. Plan proposed puncture site.
 4. Processing of CSF.
5. Trans-Isolate medium everywhere.
6. *N. meningitidis* -- proper streaking and growth on blood agar plate.
7. *S. pneumoniae* -- proper streaking and growth on blood agar plate.
8. *H. influenzae* -- proper streaking and growth on chocolate agar plate.
9. Gram stain of CSF:
 - a *N. meningitidis*: intra-cellular, Gram-negative diplococci,
 - b *S. pneumoniae*: Gram-positive diplococci, and
 - c *H. influenzae*: Gram-negative pleomorphic coccobacilli.
10. On chocolate agar plate, *H. influenzae* appear as large colourless to grey opaque colonies with no discoloration of the surrounding medium.
11. Overnight growth of *N. meningitidis* on blood agar plate appears as round, moist, glistening and convex colonies.
12. *S. pneumoniae* appear as small greyish mucoid (watery) colonies with a greenish zone of alpha-haemolysis surrounding them on the blood agar plate.
13. Presumptive identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*.
14. *N. meningitidis* (left), *S. pneumoniae* (right), and *H. influenzae* (top): (a) growth on blood agar plate and (b) growth on chocolate agar plate.
15. Identification of *N. meningitidis*.
16. Kovac's oxidase test -- positive reaction.
17. Agglutination, with clearing of the liquid, occurs when a suspension of the isolate is mixed with its homologous antiserum (left). A negative reaction, as in the case of heterologous antiserum (centre) and with saline (right), remains smooth and turbid.
18. Cystine trypticase agar-sugar reactions differentiating *N. meningitidis* from other *Neisseria* species. Acid production (yellow colour) shows oxidative utilization of dextrose and maltose with no utilization of lactose and sucrose.
19. Identification of *S. pneumoniae*.
20. Optochin susceptibility test for identification of *S. pneumoniae*. The strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the centre and lower streaks are susceptible to optochin and appear to be pneumococci.

21. The bile solubility test for two different strains. Strain 1 is not *S. pneumoniae* as both tubes are turbid. There is a slight decrease in turbidity in the tube containing bile salts for strain 1 (2nd tube from the left) but the tube is almost as turbid as the control tube (1st tube on the left). Strain 2 is *S. pneumoniae*. Note that the tube on the far right is clear, all the turbidity due to the cells has disappeared and the cells have lysed; by contrast the control tube (3rd tube from the left) is still very turbid.
22. Identification of *H. influenzae*.
23. Growth factor requirements. *H. influenzae* will grow only around the disk containing both X and V factors.
24. Growth factor requirements for *H. influenzae*: *Haemophilus* quad ID plate. This agar plate is divided into four compartments. One quadrant includes medium containing haemin (X factor) (upper left). One quadrant includes medium containing nicotinamide dinucleotide (V factor) (lower left). Another quadrant contains medium that includes both X and V factors (upper right). The fourth quadrant contains heart infusion agar with 5% horse blood (lower right).
25. Instructions for using silica gel packages for transport of isolates.
26. Quellung reaction for identification of *S. pneumoniae*.

XII. Abbreviations

ATCC	American type culture collection
BAP	Blood agar plate
CAP	Chocolate agar plate
CDC	Centers for Disease Control and Prevention
CSF	Cerebrospinal fluid
GC	Gonococcus medium
HIA	Heart infusion agar
HIA-rabbit blood	Heart infusion rabbit blood agar plate
Hib	<i>Haemophilus influenzae</i> type b
MOPS	3- (<i>N</i> -morpholino) propanesulfonic acid
NAD	Nicotinamide adenine dinucleotide
O/N	Overnight
PBS	Phosphate-buffered saline
rpm	revolutions per minute
SPS	Sodium polyanetholesulfonate
T-I	Trans-Isolate medium
TSA	Trypticase soy agar
TSB	Trypticase soy broth
WHO	World Health Organization