

Collection and Transport of Sterile-Site Specimens

Blood

Blood specimens may be obtained from patients with pneumonia, meningitis, or fever of unknown origin, among other syndromes.

Pneumonia

Blood cultures will be positive for a bacterial pathogen in approximately 10% – 35% of children with chest x-ray confirmed pneumonia. Because of the time and resources required to collect and process specimens, blood cultures should be obtained from children likely to have bacteremic pneumonia. Pneumonia should be diagnosed using criteria established by the World Health Organization (WHO): if several family members present with the same pneumonic symptoms and / or if wheezing is a major symptom, the etiology is likely to be viral and not bacterial; if the patient is a child under two years of age or a child with fever $>39^{\circ}\text{C}$, bacteremia may be easier to detect.

Meningitis

Blood cultures may be collected from a patient with meningitis when the performance of a spinal tap is contraindicated or when it is not technically feasible.

Fever of unknown origin

Blood cultures collected early after the onset of sustained fever (*i.e.*, suspected typhoid fever) may be positive for *Salmonella* serotype Typhi, a gram-negative bacillus.

Collection of blood specimens

Reference laboratories should usually receive isolates, rather than clinical specimens, but blood is a commonly collected clinical specimen, and one with which laboratorians should be familiar working.

Infection can be transmitted from patient to staff and from staff to patient during the blood-taking procedure. Viral agents pose the greatest hazard and in some instances are potentially lethal. Of particular importance are the hepatitis viruses and the human immunodeficiency virus (HIV; the virus causing acquired immunodeficiency syndrome [AIDS]). **To decrease the risk of transmission of these viral agents**, the following recommendations should be practiced.

- 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 labeled. Any containers with blood on the outside should be wiped thoroughly. Such containers should be transported in individual, sealed 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 thoroughly with soap and water, encouraging bleeding.

Report any contamination of the hands or body with blood, or any puncture wound, or any cut to the supervisor and the health service for treatment, as appropriate.

Venipuncture

An outline of the proper method for collecting blood from the arm is shown in Figure 52.

- a) Gather everything needed to complete the blood collection process: gloves, syringe, needle, tourniquet, gauze squares, cotton balls, adhesive bandage, puncture resistant container, culture medium and antiseptic; iodine tincture (100 ml of 70% isopropyl alcohol to 1 g of iodine) or povidone-iodine is

preferred, but 70% alcohol is an acceptable alternative.³⁶ 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.

Collecting a large amount of blood from a child can be difficult: 1 – 3 ml is usually sufficient, but volume of blood is directly related to culture yield. 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).

- 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 53; the most prominent vein is usually chosen for venipuncture.
- 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 the patient hold the cotton ball firmly in place until the wound has stopped bleeding. Inoculate the culture medium. Put the adhesive bandage on the wound.
- e) Use vacutainer tubes for blood collection, if they are available.

Specimens should be put into a blood-culture bottle immediately and placed in an incubator as soon as possible; if incubation is not feasible, the blood culture bottle can be kept at room temperature (20° – 25°C) for up to 8 hours. Ideally, the blood samples should be processed in a bacteriology laboratory as soon as possible after collection (*i.e.*, within 2 hours).

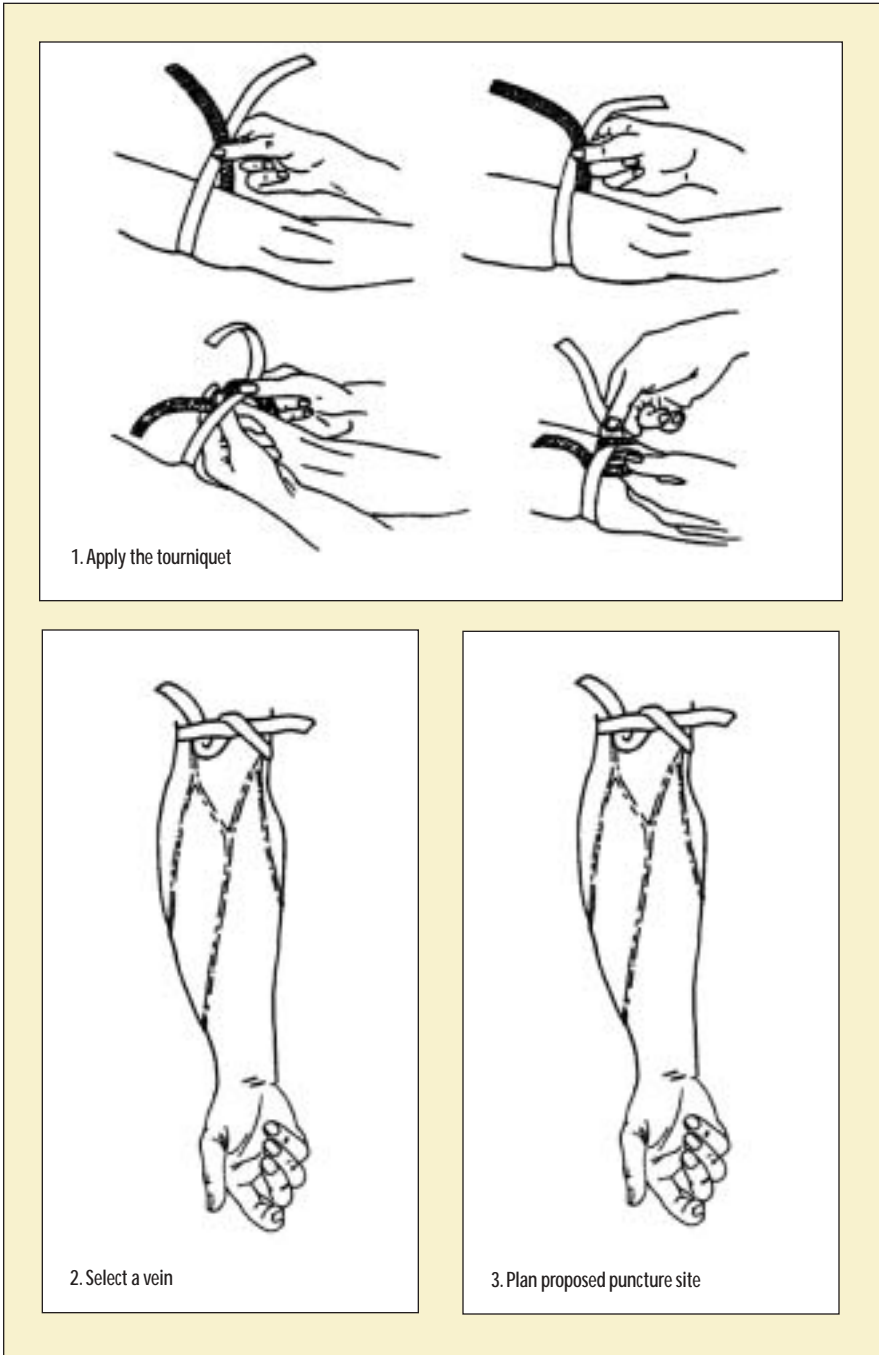
For the diagnosis of bacterial meningitis, blood should be collected when a spinal tap is contraindicated or cannot be performed for technical reasons.

Transport of blood specimens

Blood cannot be transported before being placed in broth because the collection procedure does not use an anticoagulant. If the blood-culture bottle contains a diaphragm, clean the diaphragm with 70% alcohol and povidone-iodine before inoculating the broth medium.

³⁶ Alcohol with concentrations greater than 70% has decreased bactericidal activity and should not be used.

FIGURE 53: Collection of blood from an arm



- a) Inject the blood into the broth culture medium within 1 minute of collection. The broth culture medium should contain supplemental SPS or haematin to promote survival of any organisms. 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 Appendix 2.
- b) The inoculated medium can be kept at room temperature (20°–25°C) for 4–6 hours before incubation at 35°C. **Inoculated or uninoculated blood-culture medium must not be placed in a refrigerator.** A portable incubator can be used (temperature range 25°–35°C).
- c) Immediately transport the inoculated media to the laboratory. **All inoculated blood-culture media should be received by the laboratory within 12–18 hours for subculture and should be protected from temperature extremes (<18°C or >37°C)** by using a transport carrier made of, e.g., polystyrene (e.g., Styrofoam), which can keep the samples at moderate temperature.

Cerebrospinal fluid (CSF)

If meningitis is suspected, cerebrospinal fluid (CSF) is the best clinical specimen to use for isolation and identification of the etiologic agent. Suspected agents should include *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. The collection of CSF should only be performed for diagnosis, by experienced personnel, and under aseptic conditions.

Cerebrospinal fluid (CSF) collection

Usually, three 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 (Table 25).³⁷

TABLE 25: Submission of tubes of cerebrospinal fluid (CSF) to laboratories based on the number of tubes collected from the patient

Number of tubes of CSF collected from patient	Microbiology laboratory	Chemistry laboratory	Cytology laboratory
1	Send tube 1	~	~
2	Send tube 2	Send tube 1	~
3	Send tube 2 or 3	Send tube 1	Send tube 2 or 3

³⁷ Because the presence of blood can affect cultures of CSF, it is suggested that **if more than one tube of CSF is collected from a patient**, the first tube collected (which could contain contaminating blood from the lumbar puncture) **not** be the tube sent to the microbiology laboratory.

Lumbar puncture and cerebrospinal fluid (CSF) transport

The kit for collection of CSF (Figure 54) should contain the following items:

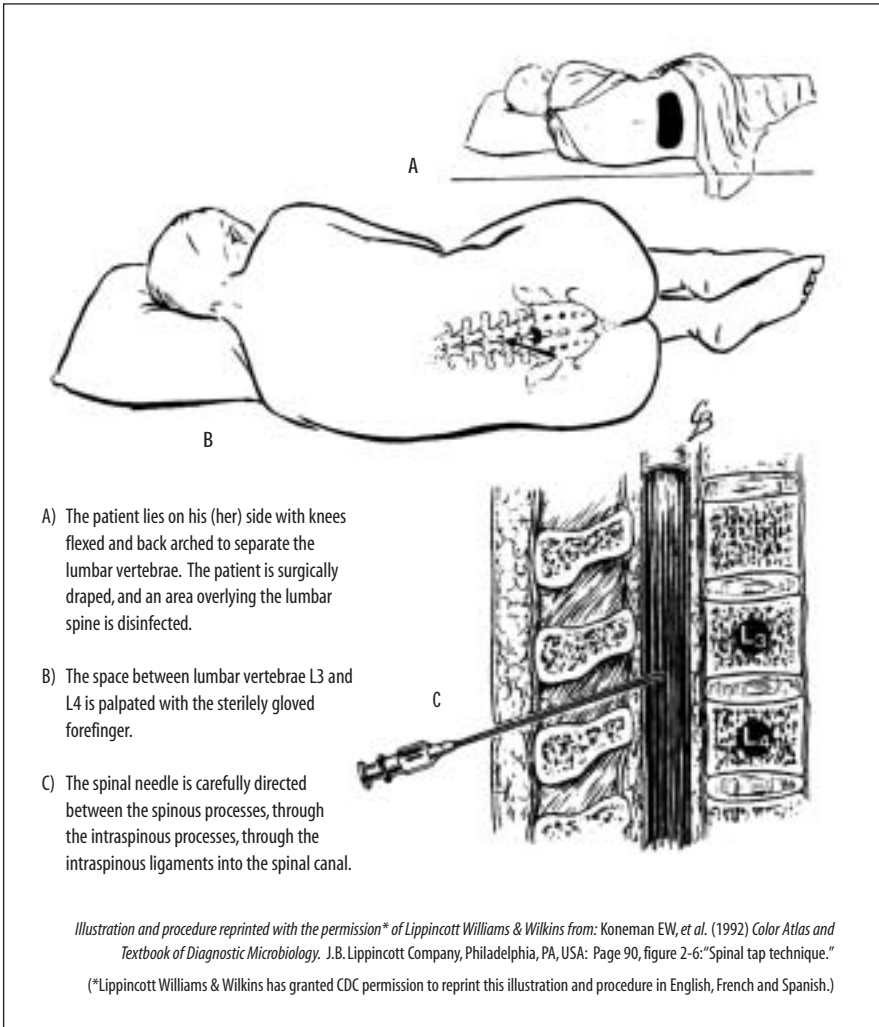
- skin disinfectant
- sterile gauze and adhesive bandages
- lumbar puncture needles: 22 gauge/3.5" for adults; 23 gauge/2.5" for children
- sterile screw-cap tubes
- syringe and needle
- transport container
- Trans-Isolate (T-I) medium (*if CSF cannot be analyzed in the microbiology laboratory immediately*)

Patients should be kept motionless for the lumbar puncture, either sitting up or laying on the side, with the back arched forward so that the head almost touches the knees during the procedure (Figure 55). 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 a tincture of iodine or povidone-iodine and let it dry. Introduce the needle is introduced, and collect the drops of fluid (1 ml minimum; 3–4 ml, if possible) into sterile, screw-cap tubes. Label the specimen with patient identification and the date and time of CSF collection.

FIGURE 54: Kit for collection of cerebrospinal fluid (CSF)



FIGURE 55: Collection of cerebrospinal fluid (CSF) by lumbar puncture



Transport of CSF specimens

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 (preferably within 1 hour from the time of collection); hand-carry the specimen to the laboratory whenever feasible. **Do not refrigerate the CSF specimen or expose it to extreme cold, and do not expose it to excessive heat or sunlight.** 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 (*i.e.*, in a CO₂-incubator or a 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 then held overnight at 35°C. T-I medium is a biphasic medium that is useful for the primary culture of meningococci and other etiological agents of bacterial meningitis from CSF (Figure 75); 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 Appendix 2.

Isolation and Presumptive Identification of Bacterial Agents from Normally Sterile Sites

General laboratories commonly receive blood samples or cerebrospinal fluid from patients with pneumonia, meningitis, or unexplained febrile illness. Laboratories may also receive urine, joint fluid, pleural fluid, or other sterile site specimens from these patients. This section of the laboratory manual provides methods for the isolation and presumptive identification of agents from these normally sterile sites. Pathogens included in this laboratory manual that could be isolated from normally sterile sites are *Haemophilus influenzae*, *Neisseria meningitidis*, *Salmonella* serotype Typhi, and *Streptococcus pneumoniae*.

Personnel who are at risk for the routine exposure to aerosolized *N. meningitidis* should strongly consider vaccination. The risk of infection when working in the laboratory with *H. influenzae* and *S. pneumoniae* is very low, and it is not required that laboratorians receive vaccination against these organisms. However, least two good vaccines (oral and injection) are available for *S. Typhi*, and laboratorians should ensure that their vaccination status remains current. Additional information on laboratory safety is included in Appendix 1.

After bacteria are recovered from normally sterile sites, the isolates require confirmatory identification; isolates received by a reference laboratory (e.g., for antimicrobial susceptibility testing) must also undergo confirmatory testing. Methods for confirmatory identification and antimicrobial susceptibility testing of *H. influenzae*, *N. meningitidis*, *S. pneumoniae*, and *S. Typhi* are presented earlier in this laboratory manual (in Chapters III, IV, V, and VII, respectively).

Blood cultures

Laboratory personnel handling blood culture specimens must be able to identify culture bottles that may have bacterial growth, isolate bacteria on solid media, and subculture isolates. Provisional identification of an isolate will often be possible on the basis of colony morphology and the microscopic appearance of a Gram-stained specimen. (Methodology for the preparation and collection of blood specimens is presented in Appendix 3.)

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. As stated in the section on specimen collection, blood cultures from young children should be diluted to 1–2 ml of blood in 20 ml of broth (1:10 to 1:20), whereas blood cultures from adults should be diluted to 5–10 ml of blood in 50 ml of broth (1:5 to 1:10). Ideally, the blood samples should be processed in a bacteriology laboratory as soon as possible after collection (*i.e.*, within 2 hours).

Inoculation of primary culture media

Blood should be cultured in a tryptone-based soy broth (commonly referred to as “Trypticase” or “tryptic” soy broth [TSB]) or brain heart infusion with a supplement, such as haematin or sodium polyanetholesulfonate (SPS). If only one blood-culture bottle is used, it should contain TSB. Neutralization of normal bactericidal properties of blood and potential antimicrobial agents is accomplished by adding chemical inhibitors such as 0.025% 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**, but it is important to use. The blood-culture bottles should be inoculated directly with blood and should be vented before incubation at 35°–37°C. Venting is accomplished by inserting a sterile cotton-plugged needle into the diaphragm (*i.e.*, rubber part) of the blood-culture bottle.

Adding growth supplements, such as IsoVitaleX or Vitox, to blood culture bottles to help support the growth of *H. influenzae* is appropriate; however, if resources are limited, a laboratory would benefit more by using this costly resource to supplement chocolate agar medium.

Identifying positive blood culture bottles

Blood-culture bottles should be examined at 14–17 hours and then every day for up to 7 days. Any turbidity or lysis of erythrocytes may be indicative of growth, and subcultures should be made immediately. Because *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* are fragile organisms, **subcultures should be routinely performed after 14–17 hours of incubation, again at 48 hours, and again at day 7, regardless of the appearance of the blood-culture bottles** because the absence of turbidity does not always correlate with the absence of bacterial growth. Before subculturing, swirl the bottle to mix the contents.

Subculture

Subcultures are made by first disinfecting the surface of the blood-culture bottle diaphragm with alcohol and a povidone-iodine swab, and then aspirating a small volume (*i.e.*, 0.5 ml) with a syringe and needle from the blood-culture bottle and inoculating the agar media with the fluid. If the bottle has a screw-cap, open the

bottle and take the fluid using sterile technique (*i.e.*, flaming the bottle mouth upon opening and closing the cap).

Ordinarily, both chocolate agar plates and blood agar plates are used for subculture. **When only one agar plate is used, it should be chocolate agar, because chocolate agar contains the X and V growth factors needed for *H. influenzae*, whereas blood agar does not.** If a blood specimen is received from a patient with a primary diagnosis of fever of unknown origin, if typhoid is suspected symptomatically, or if a Gram stain of blood-culture broth reveals gram-negative bacilli (Figure 69), add a total of 3–4 loopfuls of the blood culture onto MacConkey agar (MAC) in addition to chocolate agar and/or blood agar. Incubate the media with suspect pathogens at 35°–37°C in a 5% CO₂ atmosphere (incubator or candle-extinction jar). Because *N. meningitidis* grows well in a humid atmosphere, if an infection with *N. meningitidis* is suspected, laboratorians may choose to add a shallow pan of water to the bottom of the incubator or add a dampened paper towel to the candle-extinction jar; the moisture source should be changed regularly (*e.g.*, daily) to prevent contamination with molds.

If the laboratory has the resources to support the use of a third plate for subculture, MacConkey agar should be used, particularly when the specimen was obtained from a patient with fever of unknown origin (when typhoid fever [*S. Typhi*] or blood stream infection by gram-negative rods of other species [*e.g.*, *E. coli*, *Klebsiella*, etc.] may be suspected).

Chocolate agar should be periodically confirmed to support growth of *H. influenzae*. The agar plates should be streaked (Figures 56, 57, 58, 59a, and 59b), and incubated for up to 48 hours. The MAC and blood plates for *S. Typhi* should be incubated for 18–24 hours at 35°–37°C.

When bacterial growth has been confirmed by subculture of the blood-culture bottle, the bottle no longer requires incubation. The bottle should be disposed of according to safety procedures.

Presumptive identification of isolates from sterile-site specimens

Because the primary purpose of this section of the manual is to aid in the identification of *N. meningitidis*, *S. pneumoniae*, *H. influenzae*, and *S. Typhi* from sterile-site specimens, the methods described here will not apply to the identification of other bacterial agents (of pneumonia and meningitis) of clinical importance that are more rarely encountered. Microbiologists should refer to clinical microbiology manuals (*e.g.*, the American Society for Microbiology's *Manual of Clinical Microbiology*, the WHO's *Manual for the Laboratory Investigations of Acute Enteric Infections*, the *Clinical Microbiology Procedures Manual, Basic Laboratory Procedures in Clinical Microbiology* [WHO 2001]) or a medical microbiology manual or textbook for procedures to identify other bacteria.

FIGURE 56: Proper streaking and growth of *Neisseria meningitidis* on blood agar

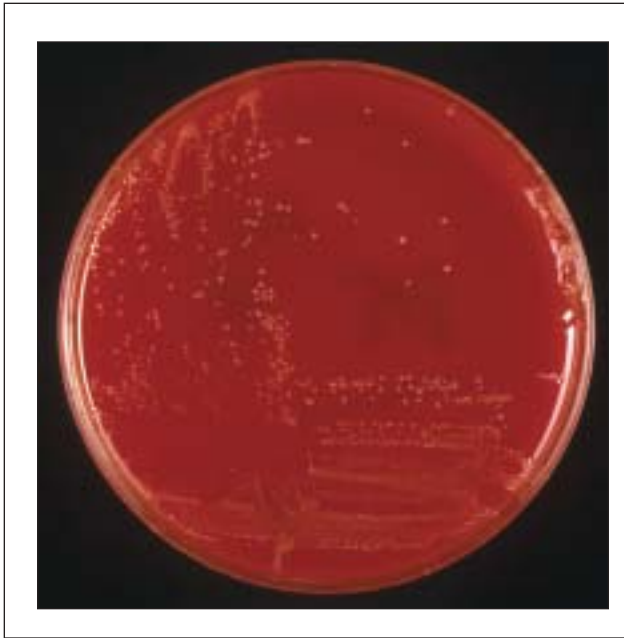


FIGURE 57: Proper streaking and growth of *Streptococcus pneumoniae* on blood agar

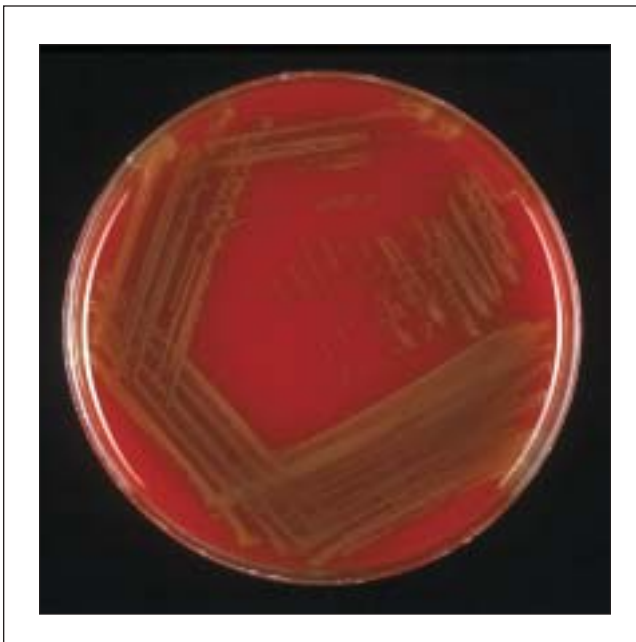


FIGURE 58: Proper streaking and growth of *Haemophilus influenzae* on chocolate agar



FIGURE 59a: Growth of *Salmonella* ser. Typhi on MacConkey agar



FIGURE 59b: Growth of *Salmonella* ser. Typhi on blood agar



Presumptive identification of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* can be made on the basis of the growth on blood agar and chocolate agar and on the basis of the microscopic morphology of the organisms (Figures 60, 61, and 62). Figure 63 provides a sample worksheet for the presumptive diagnosis of bacterial agents of meningitis and pneumonia isolated from normally sterile sites. Images comparing alpha(α)-hemolysis, alpha-prime(α')-hemolysis and beta(β)-hemolysis on sheep blood agar are shown in Figure 64.

N. meningitidis grows on blood agar, whereas *H. influenzae* will not grow without supplements (found in chocolate agar). When grown on chocolate agar, *H. influenzae* and *N. meningitidis* look similar; the two organisms can be distinguished on the agar plate by the pungent smell of indol from *H. influenzae*.

The following procedures should be followed to prepare a dried smear for Gram stain of pure culture.

- a) Place one drop of physiological saline or distilled water on an alcohol-rinsed and dried slide.
- b) With a flamed and cooled, sterile inoculating needle or loop, touch the center of the bacterial colony.
- c) Prepare a smear from the colony by adding the bacteria from the inoculating loop to the physiological saline or distilled water drop with a gentle tap. Use the loop to mix the organisms into suspension.
- d) Spread the suspension and allow it to dry, either by air (approximately ten minutes) or incubator.

Continue the Gram stain procedure with steps (c - 1) from the Gram stain methodology outlined later in this appendix. Upon microscopic examination, organisms that are gram-positive will appear violet, while gram-negative organisms will appear pink. The staining further enables the laboratorian to see morphology of the bacteria.

- **Presumptive identification of *H. influenzae***

H. influenzae appears as large, flat, colorless-to-grey opaque colonies on chocolate agar (Figure 65). No hemolysis or discoloration of the medium is apparent. Encapsulated strains appear more mucoidal than non-encapsulated strains, which appear as compact greyish colonies. Gram staining will yield small, gram-negative bacilli or coccobacilli (Figure 74). Methods for confirmatory identification and antimicrobial susceptibility testing of *H. influenzae* are included in Chapter III.

FIGURE 60: Presumptive identification of *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae*

Growth on		Gram stain morphology	Presumptive identification
Chocolate agar	Sheep blood agar		
+	+	gram-negative diplococci	<i>N. meningitidis</i>
+	+	gram-positive cocci or diplococci	<i>S. pneumoniae</i>
+	-	small, gram-negative pleomorphic coccobacilli	<i>H. influenzae</i>

FIGURE 61: Growth of *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* on sectioned blood agar and chocolate agar plates

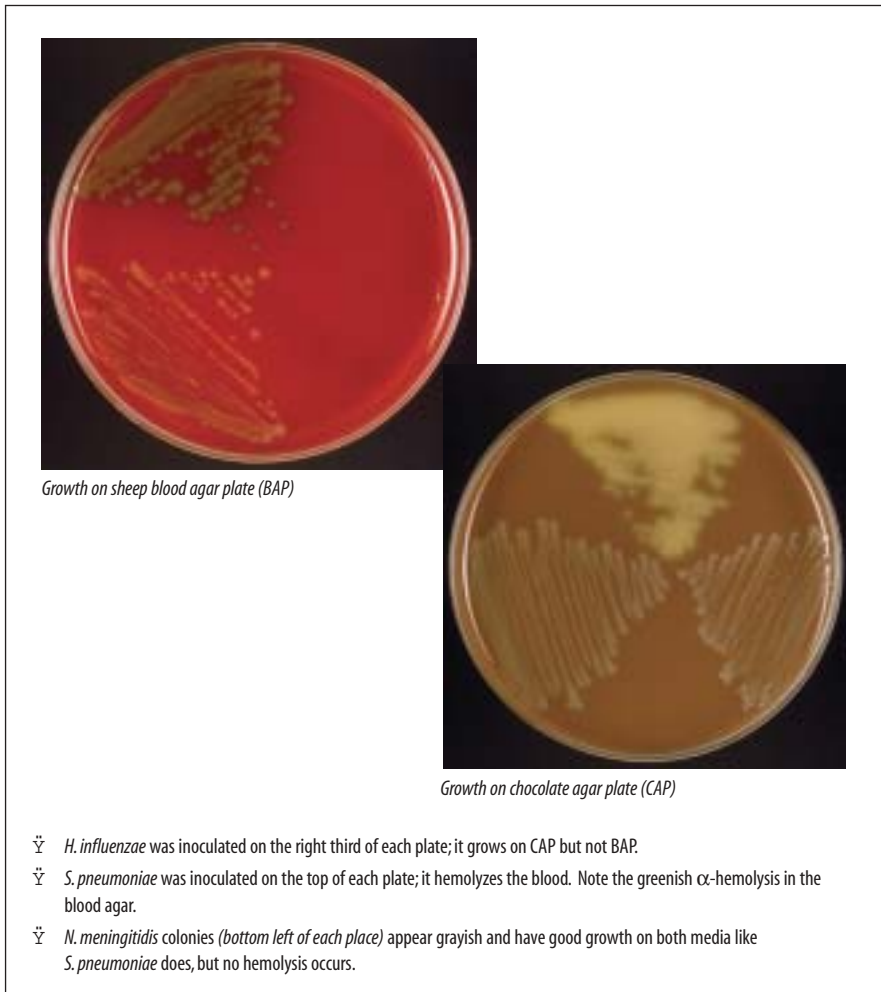
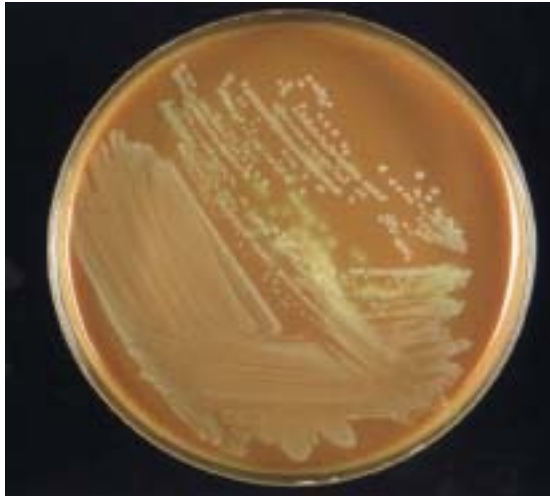
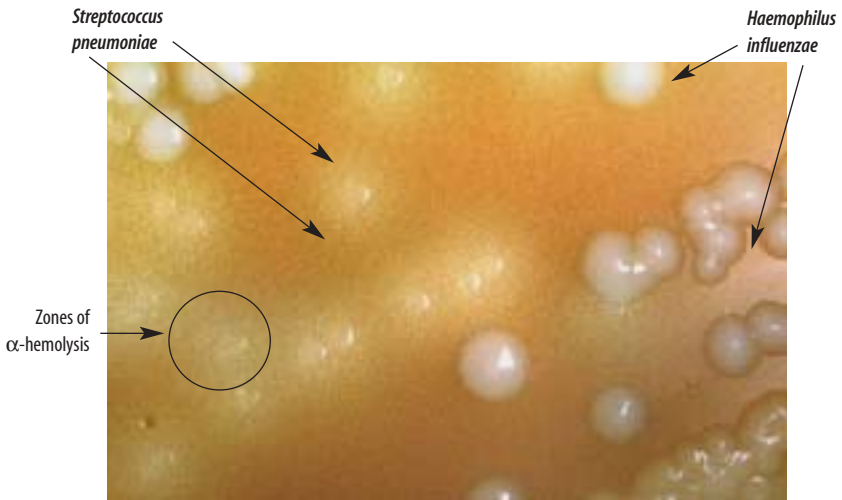


FIGURE 62: *Haemophilus influenzae* and *Streptococcus pneumoniae* colonies growing on the same chocolate agar plate



Hemolysis is apparent around the pneumococcal colonies.

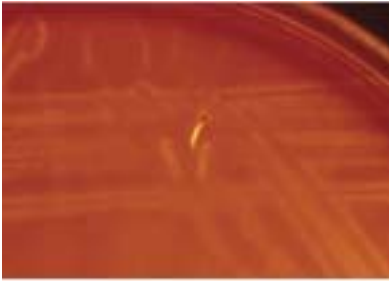


In this magnified picture, the different morphology of the colonies is easily observed. The *H. influenzae* colonies are larger and grayer than the *S. pneumoniae* colonies, which exhibit α -hemolysis.

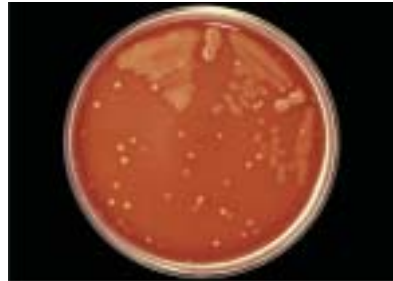
FIGURE 63. Sample worksheet for the presumptive laboratory identification of bacterial agents of pneumonia and meningitis

Specimen number, (type), date	Medium	Appearance of each colony type	Hemolysis (α , β , none)	Gram stain & arrangement	Optochin ? (If Gram + cocci)	Bile soluble ? (If Gram + cocci)	Factors? (If G — bacilli & growth on CAP only)	Oxidase test (If Gram — diplococcus)	Carbo-hydrate utilization (If Gram — diplococcus, Oxidase +)	Slide serology (H. influenzae, N. meningitidis)	Identification	
MQP4-30 (blood culture) 22-04-01	Blood agar (BAP)	BAP 1 Small, grayish Flat center	alpha	G + cocci in chains	8 mm (resistant)	N/A	N/A	N/A	N/A	N/A	Possible viridans strep	
		BAP 2 (only 1 type)	-	-	-	-	-	-	-	-	-	
		BAP 3	-	-	-	-	-	-	-	-	-	
	Chocolate agar (CAP)	CAP 1 Large, flat, mucoid	No	Small G - bacilli	N/A	N/A	Both X & V	N/A	N/A	(polyvalent +)	H. influenzae	
		CAP 2 Small, grayish flat center	alpha	G + cocci in chains	8 mm (resistant)	N/A	N/A	N/A	N/A	N/A	Possible viridans strep	
		CAP 3 (only 2 types)	-	-	-	-	-	-	-	-	-	
	Blood agar (BAP)	BAP 1										
		BAP 2										
		BAP 3										
	Chocolate agar (CAP)	CAP 1										
		CAP 2										
		CAP 3										

FIGURE 64: Alpha (α), alpha-prime (α') and beta (β) hemolysis on sheep blood agar



α -hemolysis on a streak and stab plate.
Note the light yellow-green clearing in the streaks and the stab.



β -hemolysis on a pour, streak and stab plate.
Note the clearing in the agar surrounding the colonies.



α -hemolysis surrounding a colony in a pour plate.
Note the light color surrounding the subsurface colony in the center of the image.



α' -prime hemolysis surrounding a subsurface colony in a pour plate.
Note the cells inside the clear zone of hemolysis and around the subsurface colony; a magnifying glass may assist with the visualization of α' hemolysis.

- Presumptive identification of *N. meningitidis*

On blood agar plates, young colonies of *N. meningitidis* are round, smooth, moist, glistening and convex, with a clearly defined edge. Some colonies appear to coalesce with other nearby colonies. Growth of *N. meningitidis* on blood agar 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 66). Gram staining will yield a gram negative, coffee-bean-shaped diplococcus (Figure 72). Methods for confirmatory identification and antimicrobial susceptibility testing of *N. meningitidis* are included in Chapter IV.

FIGURE 65: *Haemophilus influenzae* colonies on chocolate agar (magnified 10x)



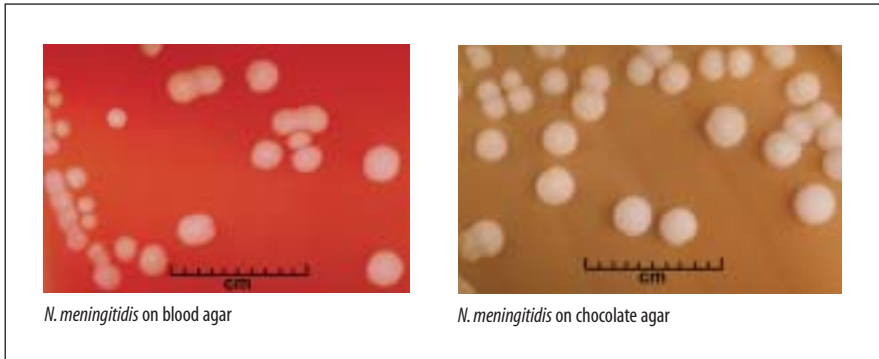
- Presumptive identification of *S. pneumoniae*

S. pneumoniae appears as small, greyish, moist (sometimes mucoid), watery colonies with a greenish zone of α -hemolysis surrounding them on blood agar (Figure 67) and chocolate agar. The degree of mucoidness of *S. pneumoniae* colonies is dependent on the freshness of the medium and the incubation atmosphere. Some serotypes appear more mucoid than others, and the fresher the medium, the more mucoid the cultures appear.

Young pneumococcal colonies appear raised, similar to viridans streptococci. Differentiating pneumococci from viridans streptococci on chocolate agar is difficult. However, a hand lens or microscope (30X-50X) is a useful aid in differentiating pneumococci from α -hemolytic viridans streptococci, which also produce a greenish zone of hemolysis on a blood- or chocolate agar plate. However, as the culture ages 24-48 hours, the colonies become flattened and the central part of each colony becomes depressed. This does not occur with the viridans streptococci (Figure 14).

Another type of colony that might appear on the culture plate along with *S. pneumoniae* is *Staphylococcus aureus* (or another *Staphylococcus* species). Figure 68 shows the two types of colonies are growing on the 5% sheep blood trypticase soy agar medium: the dull gray flat colony surrounded by a greenish zone of hemolysis is *S. pneumoniae* and the yellowish colony with no hemolytic action is *S. aureus*. Gram staining of *S. pneumoniae* will reveal a gram positive

FIGURE 66: Overnight growth of *Neisseria meningitidis* on blood agar and chocolate agar



diplococci or chain of cocci (Figure 73). Methods for confirmatory identification and antimicrobial susceptibility testing of *S. pneumoniae* are included in Chapter V.

- **Presumptive identification of *Salmonella* ser. Typhi**

Salmonella ser. Typhi grows on both blood agar and chocolate agar; on these media, *S. Typhi* colonies are grayish, transparent to opaque, glistening (shiny) and usually >1 mm in diameter. On MacConkey agar (MAC), *S. Typhi* colonies appear as colorless nonfermenters. (Colonies of *S. Paratyphi* A, *S. Paratyphi* B, and *S. Paratyphi* C and most other *Salmonella* serotypes look similar to those of *S. Typhi* on these media.) Gram staining of *Salmonella* serotypes will reveal gram-negative bacilli (Figure 69). Methods for identification and antimicrobial susceptibility testing for *S. Typhi* are included in Chapter VII.

Cerebrospinal fluid (CSF) specimens

The collection of cerebrospinal fluid (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 of isolating and identifying the etiologic agents. The collection of CSF should be performed for diagnosis only. Clinical specimens should 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.

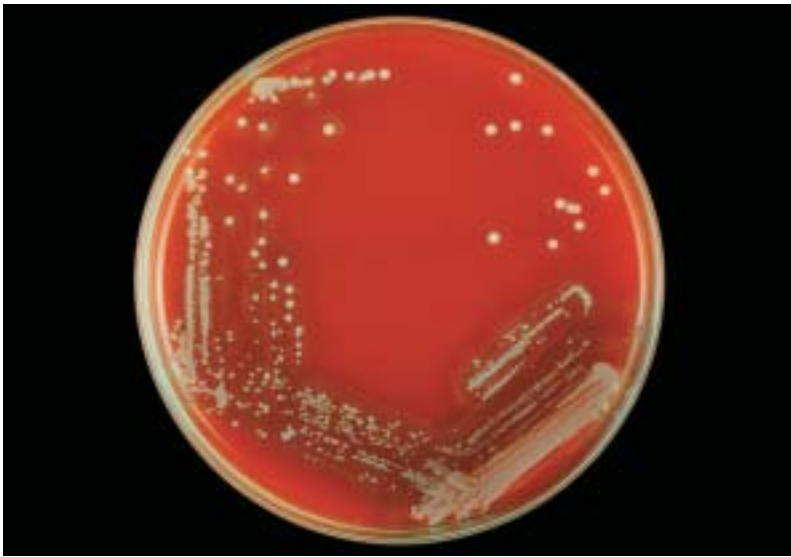
The CSF section of this manual includes only those procedures pertaining to the isolation of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* (and *S. Typhi*). Other procedures appropriate for the clinical setting and common pathogens in

FIGURE 67: *Streptococcus pneumoniae* colonies on blood agar (magnified 10x)



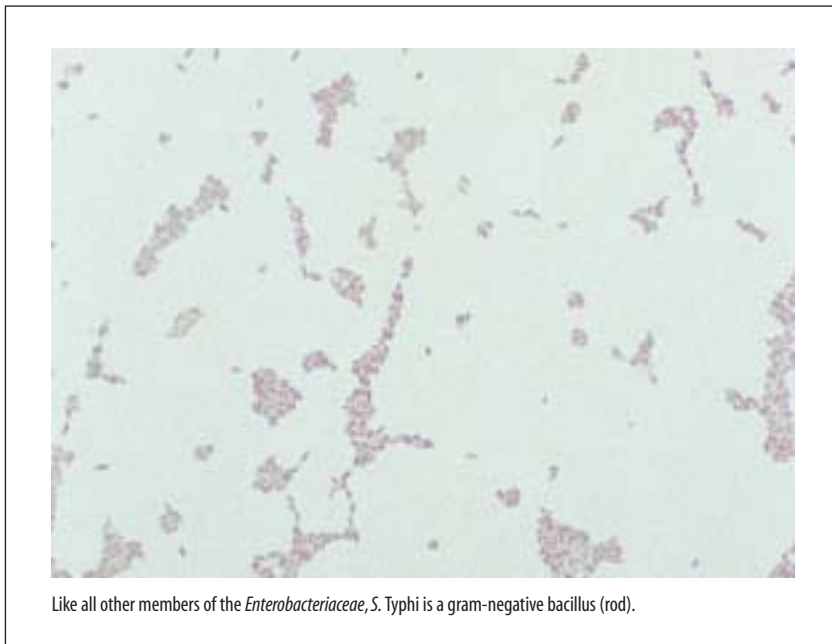
Pneumococcal colonies are mucoid and exhibit alpha-hemolysis on blood agar.

FIGURE 68: *Streptococcus pneumoniae* and *Staphylococcus aureus* growing together on the same blood agar plate



The small gray, flat colony surrounded by a greenish zone of alpha-hemolysis is *S. pneumoniae*; the gray-white-yellowish colony with no hemolytic action is *S. aureus*.

FIGURE 69: Gram stain of *Salmonella* ser. Typhi



the region may be performed on the CSF as well. These might include, but are not limited to: cell count; acid fast staining and culture for *Mycobacterium tuberculosis*; antigen detection, India ink / negative stain, or culture for cryptococcal meningitis; or others.

The contents of a kit for lumbar puncture and the procedure for collection of CSF are shown in Appendix 3. Usually, three tubes (1-ml each) 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 is available, the second or third tube should go to the microbiology laboratory (Table 25).

Primary laboratory procedures for isolation of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* from cerebrospinal fluid (CSF)

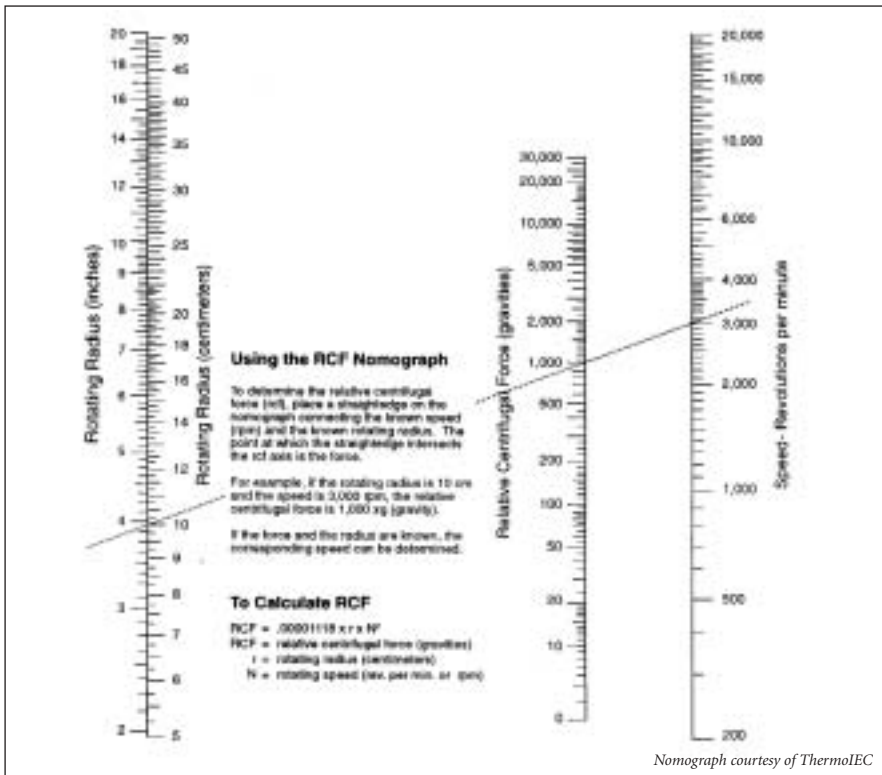
Once the CSF has arrived at the microbiology laboratory, note whether there is more than 1 ml available for analysis. **If less than 1 ml of CSF is available, it should not be centrifuged; instead, the CSF should be plated directly for the Gram stain.**

If there is >1 ml of CSF (*i.e.*, if the sample is ample for centrifugation), it must be centrifuged at a force sufficient to sediment most bacteria within 10–15 minutes; a relative centrifugal force (RCF, measured in “xg” is usually sufficient to sediment

bacteria within 10–15 minutes).³⁸ Refer to Figure 70 for a nomograph to assist in the calculation of RCF.

An algorithm for the processing of CSF specimens is presented in Figure 71. After the sample has been centrifuged, draw off the supernatant with a Pasteur pipette. (When antigen detection by latex agglutination is planned, save the supernatant.) Vigorously mix the sediment (e.g., with a vortex machine); once it is well-mixed, use one or two drops of sediment to prepare the Gram stain and use one drop to streak the primary culture media.

FIGURE 70: Nomograph for calculation of relative centrifugal force (RCF)



Presumptive diagnosis by Gram stain or latex agglutination of cerebrospinal fluid (CSF)

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

³⁸ Because centrifuges vary from laboratory to laboratory, the revolutions per minute (rpm) required should be calculated on the basis of a desired relative centrifugal force (RCF) of 1000 to sediment the bacteria within 10–15 minutes. To calculate the RCF (measured in xg), the radius of the centrifuge (radius = r) and the revolutions per minute (rpm = n) must be known: $RCF = [11.17 (r)] \times [(n / 1000)^2]$. For example, a typical bench-top centrifuge with a radius of 10.5 cm and run at 2800 rpm has a RCF of 920 xg; this RCF is sufficient to sediment bacteria in CSF in 10–15 minutes. See Figure 70 for a nomograph to assist with these calculations.

or by detection of specific antigens in the CSF by a latex agglutination test. (**Note:** Counter immunoelectrophoresis may also be used for direct antigen detection in CSF.) Positive results of either or both tests can provide evidence of infection, even if cultures fail to grow.

The Gram stain procedure for CSF (Hucker Modification)

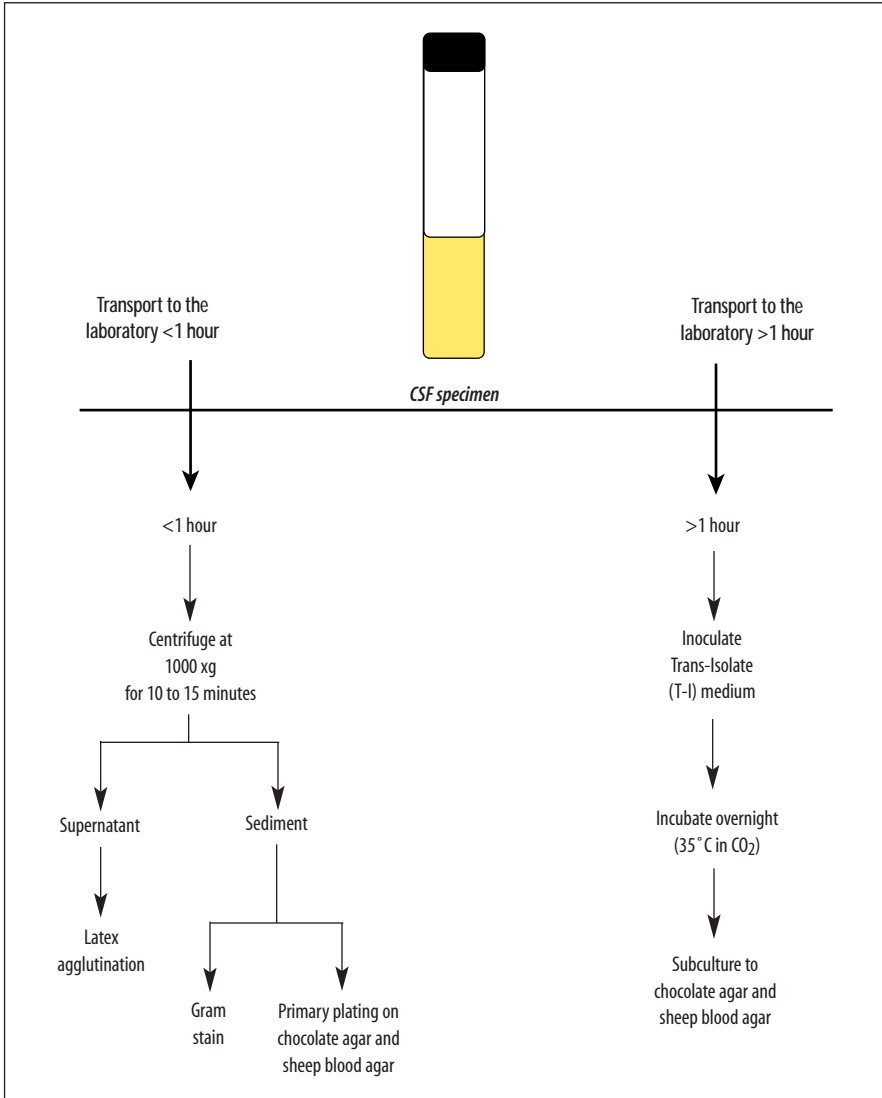
After the CSF has been centrifuged and the sediment well-mixed, a portion of the sediment is Gram stained.

- a) Centrifuge the CSF for 10–15 minutes at an RCF of approximately 1000 xg. (See footnote 38 for an explanation of this formula, and the nomograph in Figure 70 for assistance in calculating the RCF.)
 - For example, a centrifuge with a radius of 10.5cm running at 2800 rpm would yield a RCF of 920 xg. This force is sufficient to sediment out bacteria in approximately 15 minutes.
- b) Mix the sediment well, and prepare a smear by placing one or two 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) After the smear is thoroughly dry, pass the slide quickly through a flame three times to fix the smear. At this time, the slide will be slightly warm (not hot) when the back of the hand touches the bottom of the slide. Alternatively, fixation by methanol (95% – 100%) can be used for 1 minute.
- e) Flood the smear with ammonium oxalate-crystal violet and let stand for 1 minute.
- 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) **Note:** Alternatives to ethyl alcohol in this step include acetone or an ethanol-acetone mixture. If using acetone or ethanol-acetone, rinse the slide gently with water and drain.
- k) Counterstain with safranin for 20–30 seconds, or with carbol-fuchsin for 10–15 seconds.
- l) Rinse the slide with tap water. Gently blot dry with clean, absorbent tissue or paper or allow to air-dry. If using tissue or paper, it is important to blot (*i.e.*, do not rub the slide).

m) Examine the stained smear with a microscope, using a bright-field condenser and an oil-immersion lens.

Note: Some commercial Gram stain kits may have slightly different staining instructions. **It is important to use the manufacturer's instructions included with a commercial kit.**

FIGURE 71: Processing of cerebrospinal fluid (CSF)



Upon microscopic examination, organisms that are gram-positive will appear violet to blue, whereas gram-negative organisms will appear pink to red. The staining further enables the laboratorian to see morphology of the bacteria.

When examining the Gram-stained slide under a microscope, *N. meningitidis* may occur extra-cellularly or intra-cellularly in the polymorphonuclear leukocytes and will appear as gram-negative, coffee-bean-shaped (or kidney-bean-shaped) diplococci (Figure 72). *S. pneumoniae* are gram-positive diplococci sometimes occurring in short chains (Figure 73). *H. influenzae* are small, pleomorphic gram-negative rods or coccobacilli with random arrangements (Figure 74). Other manuals should be consulted for Gram stain reactions of other bacteria.

The general method for performing latex agglutination tests

Several commercial latex agglutination kits are available. 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 (at 2°– 8°C) up to several hours, or frozen at -20°C for longer periods. Reagents should be kept refrigerated at 2°– 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. General recommendations and instructions typical for the detection of soluble bacterial antigens are provided in this manual, but **follow the manufacturer's instructions precisely when using these tests.**

- 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.

The test should be read under a bright light, without magnification. The test is read as **negative** if the suspension remains homogenous and slightly milky in appearance. In contrast, the reaction is **positive** if visible clumping (*i.e.*, agglutination) of the latex particles occurs within 2–10 minutes.

Note: It is important to appreciate that false positive and **false negative reactions can and do occur with latex agglutination tests.** For example, certain proteins from *E. coli* may cross-react with *N. meningitidis* proteins in the latex agglutination test, yielding a false positive result. **Culture is therefore preferable.**

Culturing of cerebrospinal fluid (CSF)

CSF should be processed in a bacteriology laboratory as soon as possible, within 1 hour of collection. CSF should be inoculated directly onto both a supplemented

FIGURE 72: Gram stain of cerebrospinal fluid (CSF) with *Neisseria meningitidis*

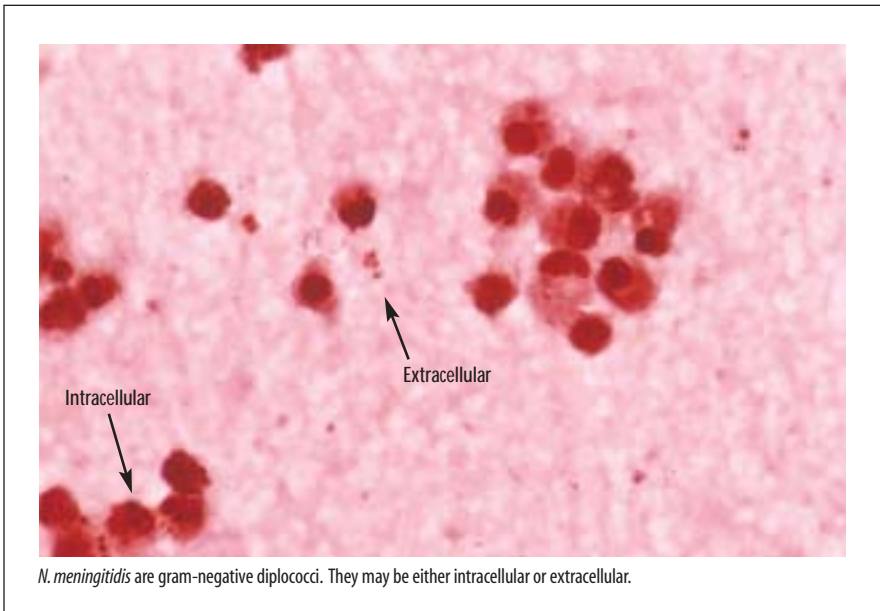


FIGURE 73: Gram stain of cerebrospinal fluid (CSF) with *Streptococcus pneumoniae*

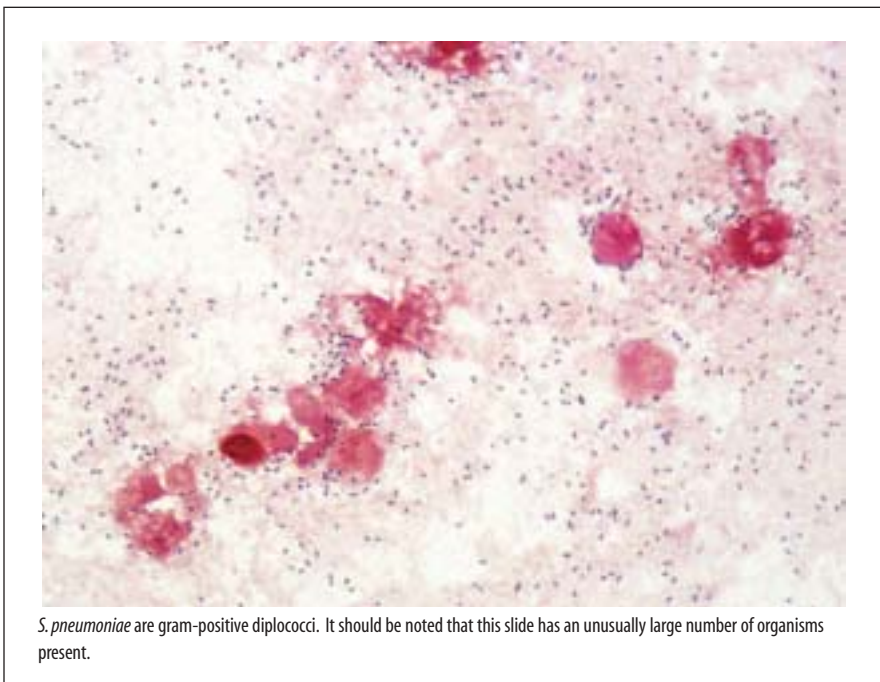
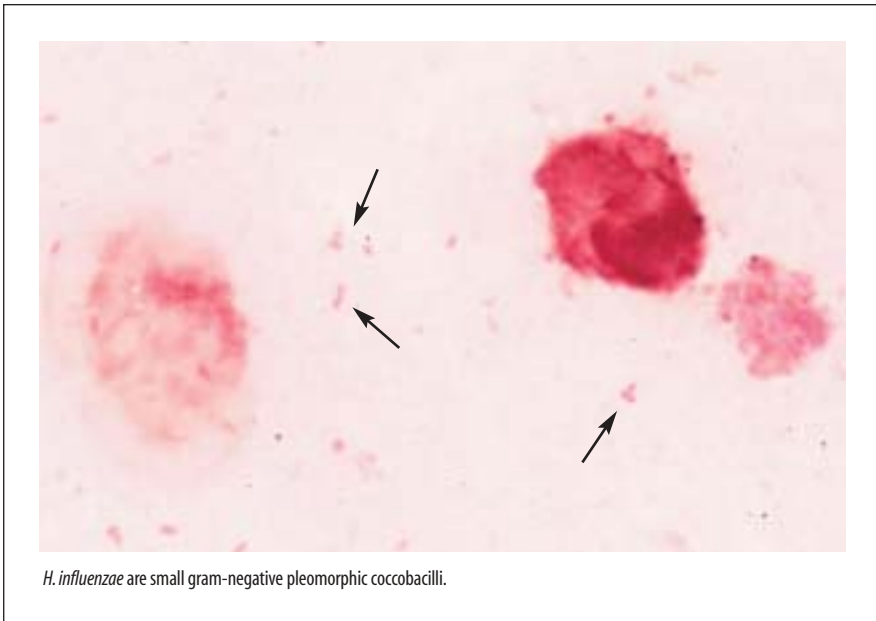


FIGURE 74: Gram stain of cerebrospinal fluid (CSF) with *Haemophilus influenzae*



chocolate agar plate and a blood agar plate. Use a sterile bacteriological loop to streak or thin the bacteria into single colonies; **the loop must be sterilized prior to each step** of the plate-streaking process.

Blood agar that has been properly streaked is shown in Figures 55, 56, and 57. The agar plates should be incubated in a 5% CO₂-incubator or candle-jar. A back-up broth (e.g., brain-heart infusion broth) should be inoculated with some of the sediment pellet and also incubated. Agar plates inoculated with CSF should be incubated in a 5% CO₂-incubator or candle-extinction jar at 35°–37°C.

The best medium for growth of *S. pneumoniae* is a blood agar plate, which is a tryptone soy agar (TSA) plate containing 5% sheep or horse blood. **Human blood is not an acceptable substitute** for the blood in the agar because the antibodies contained in human blood may inhibit bacterial growth. *S. pneumoniae* will also grow on chocolate agar.

For *H. influenzae*, a chocolate agar plate supplemented with haemin and a growth supplement (e.g., IsoVitaleX, supplement B, or Vitox) should be used. (When supplemented chocolate agar is not available, an acceptable alternative to achieve growth of *H. influenzae* on blood agar plates is achieved by cross-streaking the medium with *S. aureus*, or by applying a filter paper [or disks] saturated with X and V factors to the surface of the medium **after** the medium has 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 blood agar and chocolate agar.

If only one type of plate is available, (supplemented) chocolate agar should be used, because all three of these suspected etiological agents of pneumonia and meningitis can grow on this medium.

Appropriate utilization of Trans-Isolate medium for transport and culture of cerebrospinal fluid (CSF)

If the CSF cannot be analyzed in the microbiological laboratory immediately, Trans-Isolate (T-I) medium should be used. T-I is a biphasic medium that is useful for the primary culture of meningococci from CSF samples (Figure 75). It can be used as a growth or enrichment medium as well as a holding and transport medium for *Neisseria meningitidis*. The preparation of the T-I medium is described in Appendix 2.

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. The CSF should not be refrigerated but **held at room temperature before Gram staining.**

FIGURE 75: Trans-Isolate (T-I) medium



The T-I bottles must be labeled appropriately with the patient identification and the date and time of CSF inoculation. After inoculation, incubate the T-I bottles overnight at 35°C; alternatively, the T-I medium can be incubated 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-hour 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°–30°C). The vents must be removed before shipment. **It is essential to obtain specimens using aseptic technique and to avoid contamination when inoculating or sampling the bottles.**

When T-I medium is used for transport of CSF, after 24 hours of incubation, use a sterile needle and syringe to transfer 100 µl of the liquid portion of T-I onto the blood- and chocolate agar plates. Usually 50–100 µl is used to streak each plate, so to streak two plates draw either 100 µl or 200 µl with the syringe at one time (so that it is only necessary to go into the bottle once). Streak the plate for isolation and incubate at 35°C in a CO₂ atmosphere for up to 48 hours. (If no growth occurs, subculture the T-I medium at 3 days and again at 7 days.) Check for purity of growth by performing a Gram stain of the culture.

Presumptive identification of *H. influenzae*, *N. meningitidis*, *S. pneumoniae*, and *S. Typhi* on the basis of macroscopic examination of colonies on blood agar and chocolate agar plates is presented earlier in this chapter (see “Presumptive Identification”).

Isolation of bacterial agents from other sterile site specimens

Isolation and identification of agents in fluids from sterile site specimens can be critical in guiding patient care. When collected and processed under proper conditions, these body fluids can be good sources of some of the pathogens included in this laboratory manual, not to mention others beyond its scope.

Bone marrow

Bone marrow should be inoculated onto commercially available nutrient broth, (e.g., brain heart infusion broth or TSB). Consult a clinical laboratory manual for further, specific instructions.

Pleural fluid

Pleural fluid should be inoculated directly onto both chocolate agar and trypticase soy blood agar rather than being diluted in a broth as with blood cultures. Consult a clinical laboratory manual for further, specific instructions.

Urine

Urine is plated directly onto the appropriate medium (*e.g.*, blood, chocolate or MacConkey agar) with either 1- μ l or 10- μ l calibrated loops depending on whether the patient is suspected to have an acute urethral syndrome. Consult a clinical laboratory manual for further, specific instructions.

Middle ear fluid

Middle ear fluid is inoculated directly on appropriate medium (*i.e.* depending on the suspected agent). Consult a clinical laboratory manual for further, specific instructions.

Joint fluid

Isolation of an agent from joint fluid can be approached in several different ways (direct plating vs. amplification in a blood culture bottle vs. centrifugation and direct plating of the pellet). Consult a clinical laboratory manual for further, specific instructions.

Nasopharyngeal Swab Specimen Collection and Culture Methodology

Laboratories may receive nasopharyngeal (NP) swabs in the course of prevalence surveys and carriage studies of respiratory organisms. Culture methods for this type of specimen are included below. Once the organism has been isolated, refer to the laboratory manual section specific to that agent for antimicrobial susceptibility testing methodology.

Use swabs taken from the upper respiratory tract (*e.g.*, the nasopharynx) to inoculate the primary culture medium; the nasopharyngeal swab should be rolled over one-fourth of the plate (*i.e.*, one quadrant). Because bacteria other than *S. pneumoniae* and *H. influenzae* are generally present, selective media are used. For *S. pneumoniae*, the selective medium is a tryptone soy agar (TSA) plate containing 5% sheep or horse blood and 5 µg/ml of gentamicin sulfate; for *H. influenzae*, a chocolate agar plate containing 300 µg/ml of bacitracin is used. If one swab is being collected for recovery of both *S. pneumoniae* and *H. influenzae*, the blood agar and gentamicin plate should be inoculated first, followed by the inoculation of the chocolate agar and bacitracin plate (because *S. pneumoniae* is more susceptible to the antibacterial activity of the bacitracin than *H. influenzae* is to the antibacterial activity of gentamicin). After direct plating with the swab, use a bacteriological loop to streak the plate; Appendix 4, “Isolation of Agents from Normally Sterile Sites,” contains figures of properly streaked plates.

In areas where overgrowth of contaminants occurs in <10% of cultures, culture media without antibiotics may be used. However, in this case the primary plates **must** be streaked very carefully to allow separation of individual colonies.

Collection of nasopharyngeal (NP) swabs

NP swab collection is a clinical procedure and should therefore be performed by trained health-care workers. A specifically designed swab with a flexible wire shaft and a small calcium alginate tip should be used; calcium alginate is inert and nontoxic to *Neisseria* and other sensitive bacteria.

Figure 76 depicts the proper method of collecting an NP swab. The patient’s head should be tipped slightly backward, as shown, and immobilized. For young infants, a good way to collect NP swabs is for the person taking the specimen to hold

his/her hand behind the neck of the infant while the infant is sitting in the lap of the parent or other adult. For children, the adult should lightly hold the child's head against his/her chest with a hand on the child's forehead; the adult's other arm should be used to restrain the child's arms. Sometimes it is also helpful if the adult's legs are used to stabilize the child's legs; this reduces body movement and kicking during the collection of the NP swab.

When the child's head is immobilized and body is restrained, the NP swab can be collected using the following procedures.

- a) Unwrap the swab.
- b) Insert the swab into a nostril and pass the swab parallel to the ground, back to the posterior nares. Do not use force. The swab should travel smoothly with minimal resistance; rotating the swab during the insertion will help the swab move. If resistance is encountered, remove the swab and try the other nostril.
- c) Once in place, rotate the swab, leave in place approximately five seconds to saturate the tip, and remove slowly.
- d) Use the swab to inoculate the appropriate (selective) medium (sheep blood with gentamicin to isolate *S. pneumoniae*; chocolate agar with bacitracin to isolate *H. influenzae*; blood or chocolate with no antimicrobial for *N. meningitidis*) by direct plating, or place the swab in STGG transport medium for transportation to the laboratory.

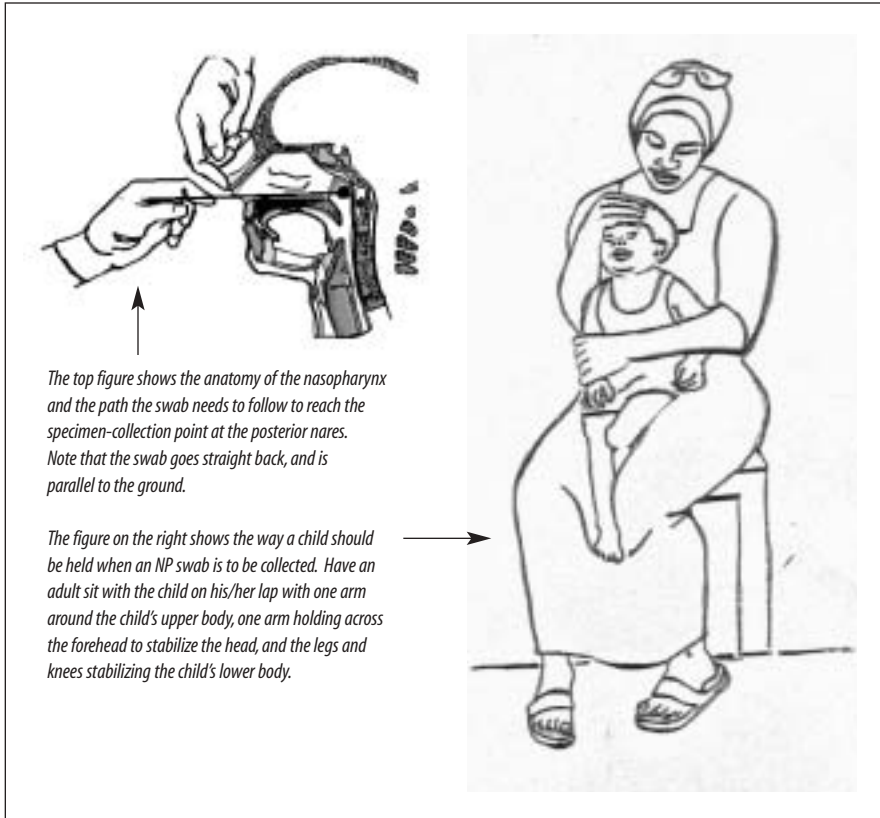
Skim-milk tryptone glucose glycerol (STGG) transport medium for nasopharyngeal secretions

Skim-milk tryptone glucose glycerol (STGG) transport medium is a tryptone broth with skim (nonfat) milk, glucose, and glycerol that can be used to transport NP swabs to the laboratory when the swabs cannot be plated directly from the patient. (The preparation of STGG medium is described in Appendix 2.) Culturing from the STGG as soon as possible is preferred, though STGG can also be used for storage and transport (for a several hours at room temperature; for up to 8 weeks at -20°C; and, for at least 2 years at -70°C).

Inoculation of STGG with an NP swab

- a) Thaw frozen tubes of STGG before use.
- b) Label the tube with appropriate patient and specimen information.
- c) Using a calcium alginate swab, collect an NP swab from the patient.
- d) Insert swab to the bottom of the STGG medium in thawed tube.

FIGURE 76: Collection of a nasopharyngeal (NP) swab



- e) Raise the swab slightly and cut the wire portion (*i.e.*, the shaft) of the swab at the top level of the container. Allow the bottom portion of the swab (*i.e.*, the tip) containing the calcium alginate material to drop into the tube.
- Discard the remaining shaft into disinfectant solution or a sharps container.
- f) Tighten the screw-cap top securely.
- *Optional:* If desired, after tightening the cap, vortex on high speed for 10–20 seconds.
- g) Freeze specimen immediately in upright position at -70°C , if possible.

In some cases, the inoculated STGG medium has been placed on ice for several hours before placing the STGG medium at -70°C without loss of viable *S. pneumoniae*. Extended storage of inoculated STGG stored at -20°C for 8 weeks results in minimal loss of viability of *S. pneumoniae*, and indications are that *H. influenzae* survive as well as *S. pneumoniae* in STGG [CDC unpublished data,

2002]. Data are not available for recovery of *N. meningitidis* from STGG. **Short-term storage of STGG is best at -70°C although a freezer at -20°C may also be used.**

Recovery of bacteria from STGG

- a) Remove the inoculated STGG medium from the freezer.
- b) Allow the tube to thaw at room temperature.
- c) Vortex each tube for a full 10 seconds.
- d) Using a sterile loop, aseptically remove a 50–100 µl sample of inoculated STGG to streak onto a plate for culture. (If attempting isolation of *S. pneumoniae*, a 100-µl inoculum is preferable.)
 - 1) **5% sheep (or horse) blood + 5 µg/ml gentamicin sulfate agar** is the appropriate plated medium for the recovery of *S. pneumoniae* from a nasopharyngeal swab specimen stored in STGG.
 - (If a gentamicin-containing medium is not available, attempt recovery from a standard blood agar plate.)
 - 2) **Chocolate + 300 µg/ml bacitracin agar** is the appropriate plated medium for recovery of *H. influenzae* from a nasopharyngeal swab specimen stored in STGG.
 - (If a bacitracin-containing medium is not available, attempt recovery from a standard supplemented chocolate agar plate.)
 - 3) 5% sheep or chocolate agar is the appropriate plated medium for recovery of *N. meningitidis*.
- e) Re-freeze the specimen (*i.e.*, the STGG) as soon as possible; keep it cool (in an ice water bath if necessary) if the time is extended beyond a few minutes at room temperature.
- f) Avoid multiple freeze-thaw cycles whenever possible. One way to decrease risk of freeze-thaw cycles within the freezer is to make sure the cryotubes are kept in the back of the freezer shelf and not the front or in the door.

Vials of inoculated STGG can be sent to other laboratories, if necessary; regulations for safe and proper packing and shipping of specimens are included in Appendix 12.

Serotyping and Quellung Typing of *Streptococcus pneumoniae*

Typing of the pneumococci isolated from patients with various clinical syndromes (e.g., sporadic cases of meningitis or pneumonia) 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. Certain studies may 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.

The Quellung reaction is traditionally used for the typing of pneumococci and is the method of choice because it is easy, fast, accurate, and economical. 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 *S. pneumoniae* stains dark blue with methylene blue and is surrounded by a sharply demarcated halo, which represents the outer edge of the capsule; 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.

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. Traditionally, a total of seven pooled sera in addition to 21 type- or group sera are needed to type or group these strains by the use of the conventional pneumococcal diagnostic antisera. 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, and types that exhibit close serological cross-reactivity are grouped together. Of the 90 types, 58 belong to 20 groups containing from two to four types; a total of 46 different pneumococcal types or groups are currently known.³⁹

³⁹ Monovalent factor sera for identification of types within groups are not discussed in this manual. However, the sera are rendered specific by multiple absorptions or by induction of immunological tolerance to cross-reacting types prior to immunization.

The procedure presented in this manual, however, describes a simple checkerboard typing system, based on 12 pooled sera and intended for typing and/or grouping most of the 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.

- a) Inoculate a freshly prepared blood agar plate with an inoculating loop. Inoculate about one-third of the plate heavily and then streak the remainder of the plate for isolated colonies. Invert the agar plate, place in a candle-jar or a CO₂ incubator and incubate at 35°C for 18–24 hours.
- b) Using a sterile loop, sweep across the surface of the 18–24 hour plate for inoculum. Prepare a light to moderate cell suspension (approximately equal to a 0.5 McFarland density standard) in 0.5 ml of physiological saline. **Optimum Quellung reactions can be observed when there are 25–50 cells visible in a microscopic field.**
- c) With a loop or micropipette, dispense 3–5 µl of pneumococcal pool antiserum and 1–5 µl of the cell suspension on a microscope slide. (Be sure not to contaminate the bottle of antisera with the cell suspension.) Add an amount of 0.3% aqueous methylene blue equivalent to the amount of antiserum, and mix the liquids on the slide.
- d) Cover the mixture with a 22-mm² cover-slip and incubate at room temperature for up to 10 – 15 minutes. **Make sure the fluid on the slide does not dry**, or it will not be possible to read the Quellung reaction.
- e) All positive Quellung reactions appear as shown in Figure 77. The capsule is seen as a clear area surrounding the dark cell (*i.e.*, the clear area between the dark cell and the dark background).

Non-reactive strains

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

FIGURE 77: A Quellung reaction



Typing and/or grouping of *S. pneumoniae* using the checkerboard system

The capsular reaction test should be performed using each of the nine traditional pools (A through I) in succession 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 26, adapted from works by Sørensen (1993) and Lalitha, *et al.* (1999) [Appendix 15]).

TABLE 26: A checkerboard typing system for *Streptococcus pneumoniae*

Existing pool ^{a,b,c}	Type or group with new pool ^{a,b,c}					Non-vaccine-related type or group ^{a,b,c}
	P	Q	R	S	T	
A	1	18*	4	5	2	
B	19*	6*	3	8		
C	7*				20	24*, 31, 40
D			9*		11*	16*, 36, 37
E			12*	10*	33*	21, 39
F				17*	22*	27, 32*, 41*
G^c						29, 34, 35*, 42, 47*
H	14	23*		15*		13, 28*
I^c						25, 38, 43, 44, 45, 46, 48

^a The five pooled sera P to T are composed such that each of the 21 vaccine-related types and/or groups reacts with both 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. (Numbers 26 and 30 are not in use.) Asterisks (*) indicate groups containing the following types: 6, 6A and 6B; 7, 7A, 7B, 7C and 7F; 9, 9A, 9L, 9N and 9V; 10, 10A and 10F; 11, 11A, 11B, 11C and 11F; 12, 12A and 12F; 15, 15A, 15B, 15C and 15F; 16, 16A and 16F; 17, 17A and 17F; 18, 18A, 18B, 18C and 18F; 19, 19A and 19B, 19C, and 19F; 22, 22A and 22F; 23, 23A, 23B and 23F; 24, 24A and 24B; 28, 28A and 28F; 32, 32A and 32F; 33, 33B, 33C and 33F; 35, 35A, 35B and 35C; 41, 41A and 41F; 47 and 47A. Types and/or groups present in the current 23-valent polysaccharide 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 checkerboard system.

Checkerboard table is adapted from Sorenson (1993) and Lalitha et al. (1999)

Antimicrobial Susceptibility Testing by Broth Microdilution

Minimal inhibitory concentration (MIC) testing by agar or broth dilution is a complex process that can be expensive and challenging to prepare, but when properly carried out its results are easily interpreted. Different bacteria may be tested in different ways (*i.e.*, using either agar or serial dilutions of the antimicrobial agent in broth). MIC tests for *Neisseria meningitidis* should be performed by broth microdilution if the Etest® is not available. **Careful preparation and quality control are extremely important in order for MIC tests to be performed accurately.**

This laboratory manual recommends the use of the Etest® antimicrobial gradient strip for MIC testing; however, if there are a large number of isolates on which to perform susceptibility testing, it may be more cost-effective to order and use commercially prepared MIC panels. Standard concentrations, or dilutions, of antimicrobial agents used in MIC testing are listed in Table 27.

***N. meningitidis*: Minimal inhibitory concentration (MIC) testing by broth microdilution**

When performing MIC testing by broth microdilution, laboratorians must first confirm the identification of the isolates as *N. meningitidis*, perform a fresh subculture, prepare a suspension equivalent to the 0.5 McFarland turbidity standard, and then use this standardized suspension to inoculate the panel of antimicrobial agents. After incubation, read, record, and interpret the results.

Preliminary examination

Examine the isolates and confirm as *N. meningitidis* prior to MIC testing.

- a) Upon receipt of the isolate(s), examine plates for purity.
- b) With a sterile disposable loop, touch the surface of one colony morphologically similar to *N. meningitidis*. Streak onto a chocolate agar plate, label the plate, and incubate at 35°C in 5% CO₂ for 18–22 hours. Because *N. meningitidis* grows

TABLE 27: Concentrations of antimicrobial agents used in minimal inhibitory concentration (MIC) testing

Standard concentration values *	Comments
0.001 µg/ml	<i>Note:</i> Different organism-antimicrobial combinations require testing with different ranges of concentrations of antimicrobial agents.
0.002 µg/ml	
0.004 µg/ml	
0.008 µg/ml	
0.016 µg/ml	<i>Note:</i> Antimicrobial gradient strips used for minimal inhibitory concentration (MIC) testing often include both the standard concentrations presented here and also concentrations at intervals between the standards.
0.032 µg/ml	
0.064 µg/ml	
0.125 µg/ml	
0.25 µg/ml	
0.5 µg/ml	<i>Note:</i> When inter-dilutional values are present: measure and record the results according to the intersection of the ellipse of growth with the test strip, as described by the manufacturer. To interpret the results, round the inter-dilutional measurement up to the next highest standard MIC concentration. (For example, an Etest® MIC of 0.096 µg/ml, but interpreted as 0.125 µg/ml for the final report.)
1.0 µg/ml	
2.0 µg/ml	
4.0 µg/ml	
8.0 µg/ml	
16.0 µg/ml	
32.0 µg/ml	
64.0 µg/ml	
128.0 µg/ml	
256.0 µg/ml	

* Standard concentrations are also commonly referred to as “dilutions”.

well in a humid atmosphere, laboratorians may choose to add a shallow pan of water to the bottom of the incubator or add a dampened paper towel to the candle-extinction jar.

- c) Examine the chocolate agar plate after incubation for isolated colonies morphologically similar to *N. meningitidis*.
- d) **Perform an oxidase test** on the morphologically suspect colonies using the swab method: gently touch a sterile swab to a suspect colony, being careful not to pick up the entire colony so that enough remains that it can be streaked to subculture if it is oxidase-positive. Using a sterile Pasteur pipette, remove a small amount of Kovac’s oxidase reagent⁴⁰ from the tube and place a drop on

⁴⁰ Some laboratories may use a different reagent, Gordon and MacLeod’s reagent (1% [wt/vol]) dimethyl-p-phenylenediamine dihydrochloride; “dimethyl- reagent”), to perform the oxidase test. The dimethyl- reagent is more stable than the tetramethyl- reagent (Kovac’s reagent), but the reaction with the dimethyl- reagent is slower than that with the tetramethyl- reagent. **If the laboratory is using the dimethyl- reagent**, a positive reaction will be indicated by a color change to blue on the filter paper (not purple, as with the tetramethyl- reagent), and **with the dimethyl- reagent it will take 10–30 minutes for a positive reaction to develop.**

the growth collected on the swab; if it turns purple, the reaction is positive for *N. meningitidis* and those specific colonies should immediately be subcultured with a sterile loop to a chocolate agar plate. Label the plate and incubate at 35°C in 5% CO₂ for 18–22 hours. Use isolated colonies from this plate to set up the antimicrobial susceptibility tests.

Ÿ If the oxidase test is negative, the isolate is not *N. meningitidis*; discard appropriately.

Inoculum preparation

- a) Prepare a suspension of the culture by touching the surface of several morphologically similar isolated colonies with a sterile cotton-tip applicator on the chocolate agar subculture plate, incubated for 18–22 hrs in 5% CO₂ at 35° C.
- b) Immerse the applicator into a tube containing sterile Mueller-Hinton broth. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix.
- c) Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard. If the turbidity is greater than the standard, dilute with broth to equal the turbidity of the standard, which will be approximately 1x10⁸ CFU/ml. (Preparation of the 0.5 McFarland turbidity standard is described in Appendix 2.)

Broth microdilution

- a) Remove a sufficient number of MIC frozen plates for testing and allow them to thaw for approximately 30 minutes.
- b) Add 2 ml of the adjusted inoculum to 38 ml of sterile distilled water.
- c) Mix well.
- d) Pour the suspension into the disposable inoculator tray, and inoculate the thawed MIC trays.
- e) Incubate the MIC trays for 18–22 hours in 5% CO₂ at 35°C.

Reading the test results

Use the following *S. pneumoniae* isolate, ATCC 49619, as a quality control strain for *N. meningitidis* antimicrobial susceptibility testing. MIC breakpoints for *S. pneumoniae* ATCC 49619 with antimicrobial agents appropriate for the treatment of infections with *N. meningitidis* are presented in Table 4 of the *N. meningitidis* chapter.

- a) Read and record the quality control results first.
- b) **If all antimicrobial agents are in control**, read the test MICs and note any trailing endpoints.

Record all information in a standard form. A sample worksheet for recording antimicrobial susceptibility results for *N. meningitidis* is included in Figure 13. *N. meningitidis* does not have breakpoints defined by NCCLS (as of 2002); interpretation of the susceptibility of a strain includes accounting for the site of the infection and the dose and pharmacokinetics of the antimicrobial agent (*i.e.*, similar to interpretive criteria laboratorians may use when performing antimicrobial susceptibility testing on other organisms without defined breakpoints), as described in the antimicrobial susceptibility testing portion of the *N. meningitidis* chapter (Chapter V) of this manual.

Specimen Collection and Primary Isolation of *Neisseria gonorrhoeae*

A schematic representation of the isolation and presumptive identification of *N. gonorrhoeae* is presented in Figure 19. For treatment purposes, using a presumptive identification is appropriate; however, for a definition of infection with *N. gonorrhoeae* to be absolutely certain, a series of confirmatory biochemical and enzymatic tests must be conducted.

N. gonorrhoeae is highly susceptible to adverse environmental conditions: strains are susceptible to extreme hot and cold temperatures and to drying. Cultures for *N. gonorrhoeae* should always be incubated at 35°–36.5°C in a CO₂-enriched, humid atmosphere. Conditions affecting the growth of *N. gonorrhoeae* are summarized in Table 28.

Specimen collection and transport

Specimens for the isolation of *N. gonorrhoeae* may be obtained from sites exposed during sexual intercourse (*i.e.*, the genital tract, urethra, rectum, and the oropharynx) or from the conjunctiva of neonates infected during birth. Details on the collection and transport of specimens are presented in Table 29. Specimens may also be obtained from Bartholin's gland, fallopian tubes, endometrium, blood, joint fluid, skin lesions or gastric contents of neonates; methods for isolation of *N. gonorrhoeae* from these less common sites are not included in this document (and laboratorians should refer to a medical microbiology procedures manual for further instruction). **Specimens for culture must not be transported on dry swabs**, but rather inoculated directly on media.

The best method for isolating *N. gonorrhoeae* is to inoculate specimens directly onto a nutritive medium and to incubate the plates immediately after inoculation at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 18–24 hours. Specimens from sites with normal flora (*i.e.*, anogenital or oro-/nasopharyngeal specimens) should be inoculated onto a selective medium such as modified Thayer-Martin (MTM), Martin-Lewis (ML), or GC-Lect® medium. Specimens from other sites may be inoculated onto a nonselective medium, such as GC-chocolate agar (*i.e.*, GC agar base, haemoglobin, and 1% defined growth supplement, as described in Appendix 2).

TABLE 28: Conditions affecting the growth of *Neisseria gonorrhoeae*

Conditions	Comments
Temperature	<i>N. gonorrhoeae</i> is sensitive to extremes of warm and cold temperatures, and requires incubation at 35°–36.5° C.
Atmosphere	<i>N. gonorrhoeae</i> strains require an increased CO ₂ atmosphere (3% – 5% CO ₂) for primary isolation. Some strains have an obligate requirement for CO ₂ , whereas other strains lose this requirement on subculture. Use a CO ₂ -incubator or a candle-extinction (candle) jar. Re-light the candle each time the candle-jar is opened to add plates. (<i>Note:</i> Vapor from scented candles can be toxic to the bacteria; therefore, only unscented candles should be used in the candle-jar.)
Humidity	<i>N. gonorrhoeae</i> is extremely sensitive to drying, and must be incubated in a humid atmosphere. To obtain this atmosphere for incubation, place a flat pan of water in the bottom of the incubator or a moistened paper towel in a candle jar. Replace the moistened paper towel daily to prevent the growth of molds, which can contaminate cultures. Periodically, decontaminate the candle jar.
Growth medium	<i>N. gonorrhoeae</i> is a fastidious organism which requires supplements for growth. The growth medium recommended for <i>N. gonorrhoeae</i> is a GC-base medium containing a 1% defined supplement (IsoVitaleX or Kellogg's defined supplement).
Time	<i>N. gonorrhoeae</i> will usually survive for 48 hours in culture, but isolates should be subcultured every 18–24 hours for maximum viability. An 18- to 24-hour culture should be used to inoculate any culture-based test.
Storage	For long-term storage, strains of <i>N. gonorrhoeae</i> should be suspended and frozen in a medium such as trypticase soy broth containing 15% glycerol. Freeze the suspensions in liquid nitrogen or in a -70° C freezer. Strains do not survive for more than a short time (a few weeks) at -20° C.
Swab materials	<i>N. gonorrhoeae</i> is sensitive to some materials found in swabs. If gonococcal growth is sparse, consider that the swab material may be toxic. Some untreated cottons can be toxic to <i>N. gonorrhoeae</i> , as can the wooden-stick applicator if it is in contact with the bacteria for an extended period of time. However, laboratorians should not use only swabs made from synthetic materials for two reasons: <ol style="list-style-type: none"> 1) synthetic swabs often do not absorb liquid easily; and, 2) synthetic swabs have flexible plastic applicators. When these are pressed against the side of a tube or plate to express liquid they can splatter the suspension, which may cause laboratory-acquired infections. For this reason, laboratorians working with flexible-handled swabs should wear safety goggles.

If specimens must be transported from the point of specimen collection to a local laboratory and the inoculated media cannot be incubated during the period before transport, transporting the inoculated plates in a CO₂-enriched atmosphere is more important than incubating them at 35°–36.5° C. Inoculated media may be held at room temperature in a CO₂-enriched atmosphere in candle-extinction jars or an alternative CO₂-generating system for up to 5 hours without appreciable loss of viability; however, if the specimen is going to be transported to a distant

laboratory, it should be incubated for 18–24 hours at 35°–36.5°C in a CO₂-enriched, humid atmosphere **prior to transport**. When specimens must be transported to distant laboratories, they may be inoculated onto transport systems such as Jembec® plates (which contain a CO₂-generating system), Transgrow bottles, or agar slants containing a gonococcal selective or nonselective medium. All inoculated specimens should be delivered to the laboratory within 24–48 hours of collection to maximize recovery of gonococcal isolates.

Nutritive or buffered non-nutritive semisolid transport media (e.g., Stuart's or Amies media) have been used to transport specimens on swabs to laboratories.

Although gonococci may survive in these media for 6–12 hours, viability decreases rapidly thereafter and isolates may not be recovered after 24 hours.

In addition, because the specimen may be diluted in the transport medium, recovering isolates from semisolid transport media may be more difficult than recovery from solid agar media. When commercially available zipper-locked, CO₂-generating systems (such as Jembec®) are available, it is no longer recommended that specimens for the isolation of *N. gonorrhoeae* be transported in semisolid transport media.

Incubation conditions

***N. gonorrhoeae* requires a CO₂-enriched atmosphere for primary isolation.**

Although some strains lose their requirement for a CO₂-enriched atmosphere for growth in subculture, some strains have an obligatory requirement for CO₂ which is not lost on subculture. CO₂ incubators should be used if large numbers of specimens must be processed. If a CO₂ incubator is not available, culture plates may be incubated either with commercial CO₂-generating systems (producing a concentration of 3%–5% CO₂) or in candle-extinction jars. To use a candle-extinction jar:

- a) Place the plates to be incubated into the jar and place a small candle into the jar on the bottom, beside the plates. (The candle can be placed atop the plates, but only if the jar's top is not made of plastic, which can melt and/or produce toxic fumes when exposed to a flame.)
- b) Light the candle, and place the lid on the jar. The flame will soon self-extinguish.

When the candle-flame extinguishes from lack of oxygen, an atmosphere of ~3%–5% CO₂ has been generated. Because the vapor from scented candles may be toxic, it is important to use a non-scented candle in the candle-extinction jar. **Relight the candle each time the jar is opened to add more plates.**

Gonococcal strains also require increased humidity for good growth. Humidity is maintained in incubation chambers by placing a pan of water on the bottom shelf of a CO₂ incubator or by placing moistened but not dripping paper towels on

TABLE 29: Specimen collection procedures for the laboratory diagnosis of *Neisseria gonorrhoeae*

Specimen	Procedure	Special notes
Urethra (male)	<ol style="list-style-type: none"> 1. Insert a urethrogenital swab (rayon or Dacron*) 2–4 cm into the urethral lumen, rotate the swab and leave it in place for at least 2 seconds to absorb the fluid. (* Do not use a cotton swab unless it's been treated by the manufacturer to neutralize toxicity.) 2. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO₂-enriched atmosphere or place in a candle-extinction jar for transport to the laboratory. 3. Prepare a smear for Gram stain. 	<ol style="list-style-type: none"> a. Specimen should be collected no sooner than 1 hour after the patient has urinated. b. A presumptive laboratory diagnosis of gonorrhea may be made immediately by Gram stain (or Loeffler's methylene blue). A high correlation exists between the observation of gram-negative diplococci in Gram stained smear and the isolation of <i>N. gonorrhoeae</i> from the male urethra. c. Clean-catch, midstream urine specimens (5–10 ml) should be centrifuged and the sediment should be inoculated onto a selective medium for the isolation of <i>N. gonorrhoeae</i>.
Cervix	<ol style="list-style-type: none"> 1. Insert a non-lubricated speculum into the vagina so the cervix can be seen. 2. Use a swab to remove mucus and secretions from the cervical os; discard this swab. 3. Use a sterile swab to gently but firmly sample the endocervical canal. 4. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO₂ environment or place in a candle-extinction jar for transport to the laboratory. 	<ol style="list-style-type: none"> a. Ensure that the swab used to collect the endocervical specimen does not touch the vaginal walls during the procedure. b. In pre-pubescent girls, vaginal specimens may be substituted for endocervical specimens.
Vagina (only in prepubescent females)	<ol style="list-style-type: none"> 1. Wipe any excessive secretions or discharge. 2. Rub a Dacron or rayon swab against the mucus membranes of the posterior vaginal wall for 10–15 seconds to absorb secretions. 3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO₂ environment or place in a candle-extinction jar for transport to the laboratory. 	<ol style="list-style-type: none"> a. Collect the specimen from the vaginal orifice if the hymen is intact.

TABLE 29: Specimen collection procedures for the laboratory diagnosis of *Neisseria gonorrhoeae*, continued

Specimen	Procedure	Special notes
Rectum (if a history of oral-genital or anal-genital exposure exists)	<ol style="list-style-type: none"> 1. Insert a sterile swab approximately 1 inch beyond the anal sphincter. 2. Gently rotate the swab to sample the anal crypts. 3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO₂ environment or place in a candle-extinction jar for transport to the laboratory. 	<ol style="list-style-type: none"> a. Discard anorectal swabs that are contaminated with fecal material; obtain a second specimen.
Pharynx (if a history of oral-genital or oro-anal exposure exists)	<ol style="list-style-type: none"> 1. Depress tongue with a tongue depressor. 2. Use a sterile swab to sample the posterior pharynx, tonsils, and inflamed areas. 3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO₂ environment or place in a candle-extinction jar for transport to the laboratory. 	—
Conjunctiva	<ol style="list-style-type: none"> 1. Moisten two swabs with sterile saline. 2. Swab each eye with a separate swab by rolling the swab over the conjunctiva. 3. Immediately inoculate each swab onto a non-selective plate (e.g., chocolate agar) and incubate inoculated plates immediately in a CO₂ environment or place in a candle-extinction jar for transport to the lab. 4. Smear each swab onto a separate slide for Gram stain. 	<ol style="list-style-type: none"> a. Sample both conjunctivae if possible, but if prohibitively expensive, culture infected conjunctiva. b. <i>Neisseria</i> spp. other than <i>N. gonorrhoeae</i> (e.g., <i>N. cinerea</i> and <i>M. catarrhalis</i>) may infect the conjunctiva, particularly in newborns. Therefore, confirm the identification of gram-negative diplococci to eliminate non-gonococcal species. c. Gram-negative diplococci may be isolated after prophylactic treatment of newborn conjunctiva to prevent <i>N. gonorrhoeae</i> infection. <i>N. cinerea</i> is less susceptible to erythromycin than <i>N. gonorrhoeae</i>.

the bottom of the candle-extinction jar. Replacing the moistened towels each time that the candle-extinction jar is opened is not necessary; however, the towels should be replaced at least once a week to ensure that they do not become a source of contamination, particularly with molds.

Gonococcal isolates should not be expected to survive for >48 hours in culture, although some isolates may survive for 72–96 hours. Isolates should be subcultured every 18–24 hours to maintain maximum viability. Similarly, isolates that are stored by freezing or lyophilization should also be subcultured at least once after the initial recovery culture before being used to inoculate tests. Diagnostic tests requiring viable organisms and antimicrobial susceptibility tests must be inoculated only with cultures 18–24 hours old.

Consideration for cultures received at the laboratory at the end of the work-week

In some circumstances, a culture is growing but has not been purified by the end of the work-week, or a culture-slant has been received at the end of the work-week and personnel are not available to perform laboratory testing on it for several days. In this scenario, the best procedure for recovery of gonococci is to scrape the growth off the culture medium, avoiding visible contaminants, and prepare it for short-term storage (as described in Appendix 11). Freeze the isolate in glycerol-trypticase soy broth, and then thaw the culture at the beginning of the next work-week, when testing resources are available. (Appendix 11 also includes methods for culturing isolates from frozen cultures.) Although it may seem labor-intensive to prepare and store the isolate in a freezer for only two days, this process is more favorable for the recovery of *N. gonorrhoeae* than just letting it grow on the original medium over the weekend and then trying to recover it from the original slant.

Primary specimen swabs received at the end of the workweek should be plated onto medium appropriate for the specimen collection site (Table 29) and placed into an incubator at 35°–36.5°C in a CO₂-enriched, humid atmosphere. Although the organism might not be viable on the next workday, performing a Gram stain and an oxidase test for presumptive identification of the growth on the culture plate may still be feasible (because neither of these presumptive diagnostic tests requires viable growth).

Culture: Specimen inoculation and isolation

- a) Warm plates of selective or nonselective medium (as appropriate for the anatomic site of specimen collection, see Table 29) to room temperature.
- b) Inoculate specimens onto pre-warmed plates using the ‘Z’ streak inoculation method (Figure 78). Incubate inoculated plates at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 18–24 hours.

- c) Examine the plates after incubation. *N. gonorrhoeae* produces different colony types, which vary in diameter from 0.5 to 1.0 mm. In primary cultures, most colonies will be 0.5 mm in diameter, although a few colonies of 1.0 mm may be present. Typical colonial morphology is described in Table 30 and pictured in Figure 79.

If colonies are observed after incubation for 24 hours, use an inoculating loop to harvest growth of several colonies and streak the growth for isolation on a GC-chocolate agar plate to obtain a pure culture. Incubate the plate at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 18–24 hours.

Note: If only one or two colonies are present on the primary isolation plate, streak a portion on a GC-chocolate agar plate for subculture, but also ‘re-streak’ each colony over a small section of the primary isolation plate. Incubate both plates at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 18 – 24 hours. The primary isolation plate can be discarded if the colony subcultured onto the GC-chocolate agar plate grows successfully.

If no colonies are observed on the primary isolation plate after incubation for 24 hours, re-incubate the plate and examine it after an additional 24 hours (*i.e.*, after a total of 48 hours). If growth is still not observed on the primary isolation plate, this step should be repeated again. If no colonies are present after incubation for a total of 72 hours, the specimen should be reported as having “no growth.”

If colonies exhibit morphology typical of *N. gonorrhoeae* (Figure 79), continue with a Gram stain or simple single stain (*e.g.*, Loeffler’s methylene blue stain) for cellular morphology.

Gram stain (or simple stain with Loeffler’s methylene blue, safranin, or malachite green)

The morphology of the gonococcus can be demonstrated with a Gram stain or, alternatively, with a simple stain using Loeffler’s methylene blue, safranin, or malachite green. Although *N. gonorrhoeae* is a gram-negative diplococcus with a characteristic flattened coffee-bean shape, because of the way cells divide, they may also appear as tetrads or clumps when stained. Images showing results of a typical Gram stain and simple Loeffler’s methylene blue stain of *N. gonorrhoeae* are presented in Figure 80. Performance of the Gram stain is described earlier in this laboratory manual, in Appendix 4 (“Isolation of Agents from Normally Sterile Sites”). Smears for the Gram stain may be prepared from a specimen swab, individual colonies on the primary isolation medium, or from pure culture.

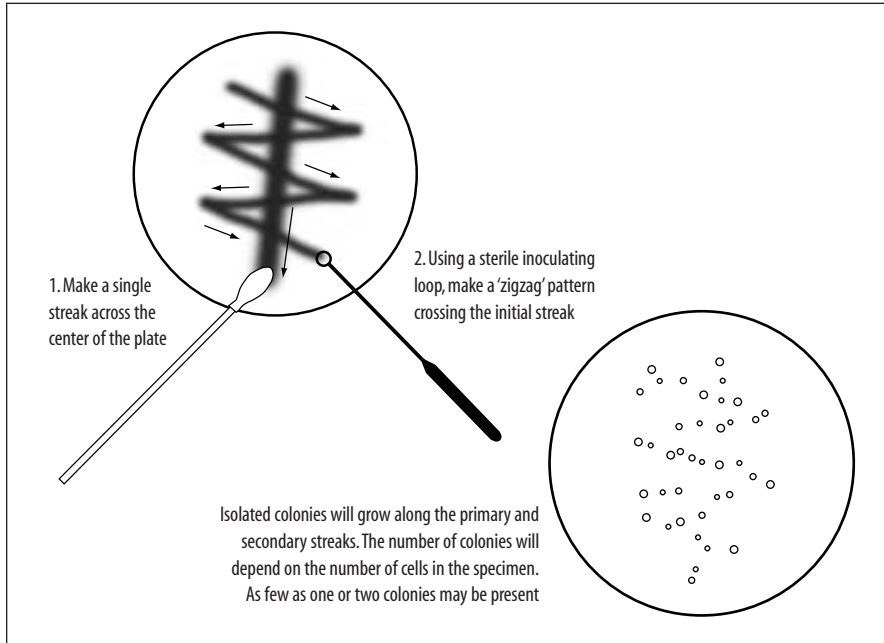
It should be noted that, when Gram staining, clumps of cells might appear a dark color due to the retention of crystal violet in the clump, even after proper decolorization, leading to the misinterpretation of some gram-negative cells as gram-positive. However, attempts to adequately decolorize the clumps may result

TABLE 30: Colonial morphology of *Neisseria gonorrhoeae* and related species on gonococcal selective media

Species	Comments
<i>N. gonorrhoeae</i>	<ul style="list-style-type: none"> Colonies are of similar appearance on gonococcal selective and nonselective media: pinkish-brown and translucent, 0.5–1.0 mm in diameter, smooth consistency, and defined margins. <ul style="list-style-type: none"> 0.5-mm colonies tend to be high-convex in elevation 1.0-mm colonies tend to be low-convex in elevation Fastidious strains of <i>N. gonorrhoeae</i> produce atypically small, “pinpoint” colonies (~0.25 mm in diameter) compared with other, less fastidious gonococcal strains.
<i>N. meningitidis</i>	<ul style="list-style-type: none"> Colonies are of similar appearance on gonococcal selective and nonselective media: pinkish brown and translucent, with smooth consistency and defined margins Colonies are usually larger and flatter than those of <i>N. gonorrhoeae</i> (1.0– 2.0 mm for <i>N. meningitidis</i> colonies vs. 0.5–1.0 mm for <i>N. gonorrhoeae</i>). Colonies of encapsulated serogroups A and C strains may be mucoid.
<i>N. lactamica</i> <i>N. cinerea</i> <i>N. polysaccharea</i> <i>K. denitrificans</i>	<ul style="list-style-type: none"> Colonies are of similar appearance to <i>N. gonorrhoeae</i> on gonococcal selective and nonselective media: pinkish-brown and translucent, 0.5 – 1.0 mm in diameter, low-convex in elevation, smooth consistency, and defined margins. <ul style="list-style-type: none"> Colonies of <i>N. lactamica</i> may have a yellowish pigment. Colonies of <i>N. cinerea</i> may have a brownish pigment.
<i>N. subflava</i> biovars <i>N. sicca</i> <i>N. mucosa</i>	<ul style="list-style-type: none"> Colonies are usually 1.0–3.0 mm in diameter, opaque, and may have yellow pigment (especially <i>N. subflava</i> biovars). <ul style="list-style-type: none"> Colonies of <i>N. subflava</i> bv. <i>perflava</i> and <i>N. mucosa</i> are convex and glistening. Colonies of <i>N. subflava</i> bv. <i>subflava</i> and <i>flava</i> are low-convex to flat with a matte surface and may have a slightly brittle consistency. Colonies of <i>N. sicca</i> may adhere to the agar surface and become wrinkled with prolonged incubation.
<i>M. catarrhalis</i>	<ul style="list-style-type: none"> Colonies are usually 1.0–3.0 mm in diameter, opaque, friable (dry) in consistency, and pinkish-brown. <ul style="list-style-type: none"> Colonies may be moved intact over the surface of the medium with an inoculating loop. Colonies disintegrate in chunks when broken with a loop.

in over-decolorization of the Gram stain, which could render gram-positive organisms to falsely appear gram-negative. Because the division of gonococcal cells may cause them to smear in clumps, as noted above, they can be technically complicated to stain. As a result, when staining is being performed **specifically to detect the gonococcus**, some laboratorians may find it preferable to perform a simple stain with Loeffler’s methylene blue (or another stain such as safranin or malachite green) to reveal information about the characteristic cell morphology and arrangement.

FIGURE 78: Streaking a plate with a specimen swab for primary isolation of *Neisseria gonorrhoeae*: “Z-streak” inoculation method



Methods to perform a simple stain with Loeffler's methylene blue (or safranin or malachite green) are presented below.

- With an inoculating loop or sterile swab, touch a representative colony with morphology typical of gonococcus on the primary isolation plate. The advantage of using a sterile swab for the preparation of this smear is that an oxidase test can be performed directly on the growth remaining on the swab after smear preparation.
- Prepare a thin smear of the suspect colony in a drop of water on a clean microscope slide (as for a Gram stain).
- Heat-fix the smear (as for the Gram stain).
- Cover the smear with methylene blue stain (or safranin or malachite green) for 30–60 seconds.
- Rinse and blot the slide until dry.
- View the stained smear under the oil immersion lens of a light microscope.
- Record results.

FIGURE 79: Colonial morphology typical of *Neisseria gonorrhoeae*

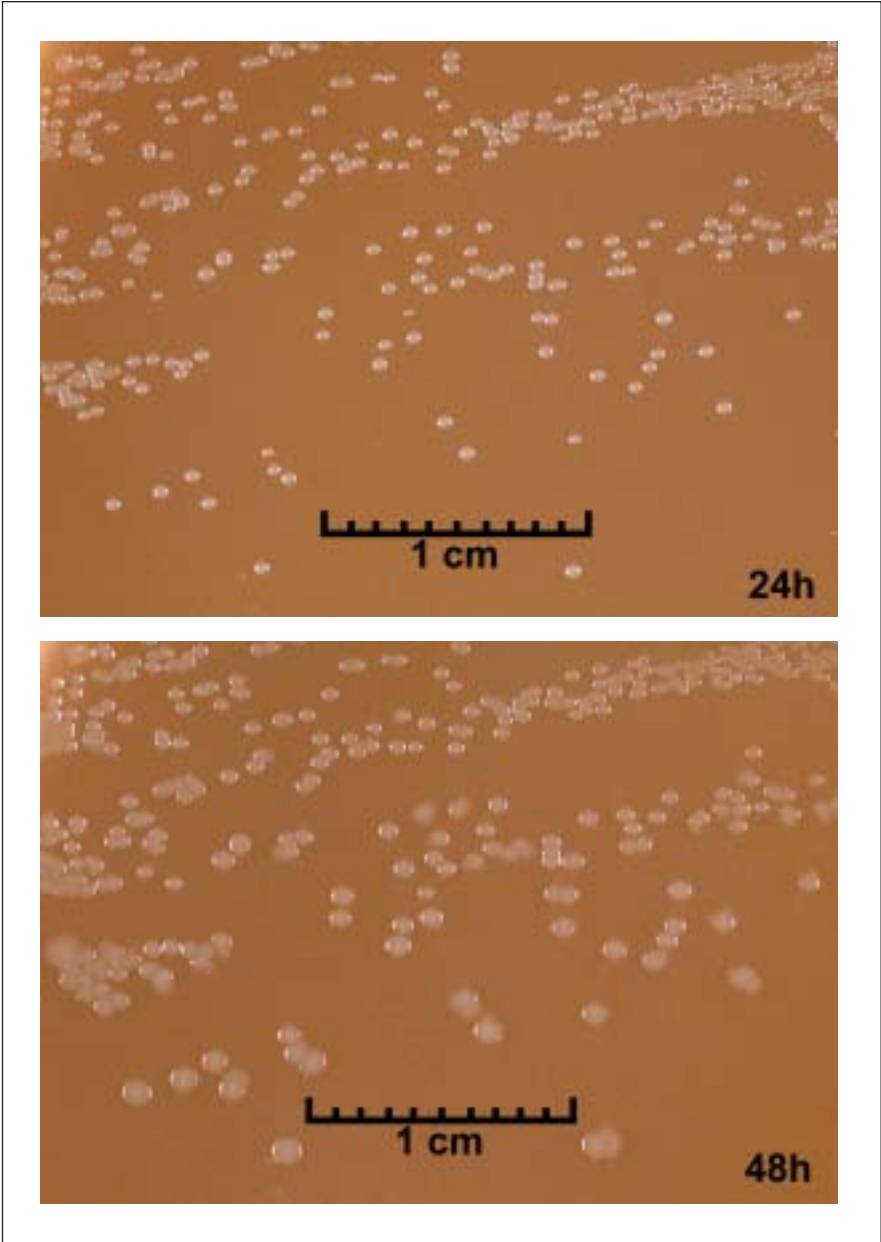
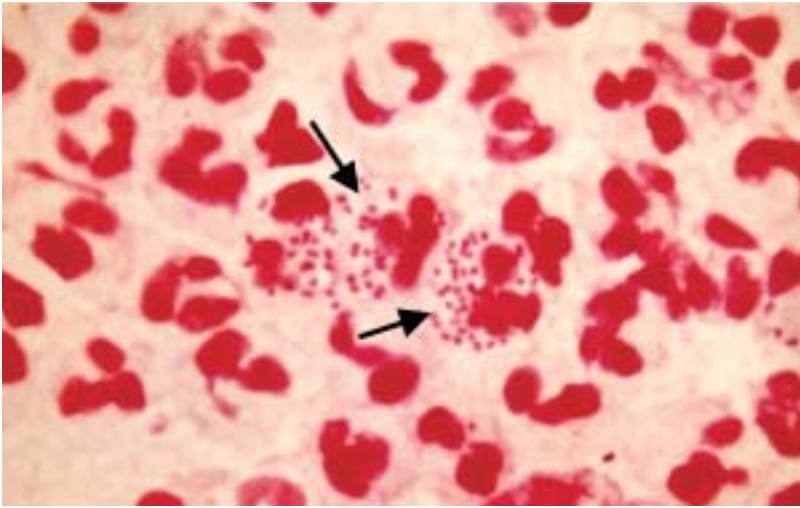
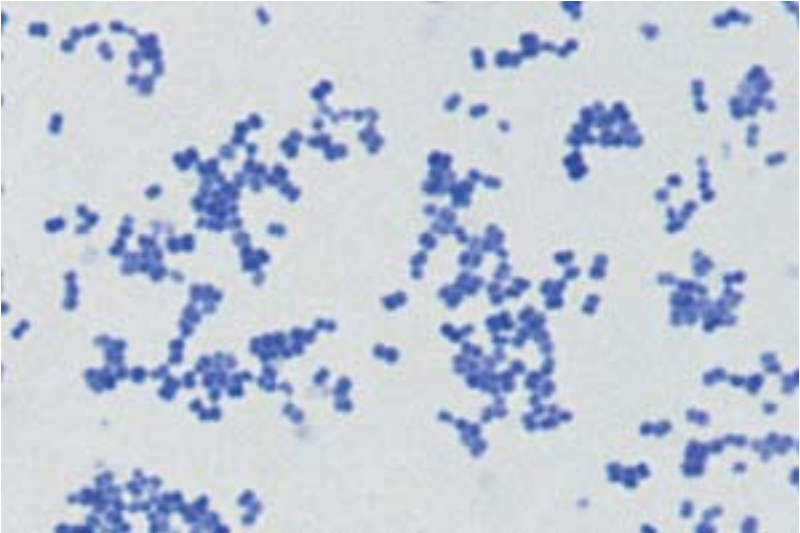


FIGURE 80: Gram stain and simple single Loeffler's methylene blue stain of *Neisseria gonorrhoeae*



A. Gram stain (of clinical specimen)



B. Loeffler's methylene blue stain (of pure culture)

Arrows point to gram-negative diplococci surrounded by polymorphonuclear neutrophils in a typical Gram stained smear of *N. gonorrhoeae* in a clinical specimen (A). The characteristic flattened coffee-bean cellular arrangement is also readily apparent if the culture is stained with a simple single stain only, such as Loeffler's methylene blue or safranin (B, simple stain of pure culture).

If colony and cell morphology are characteristic of *N. gonorrhoeae*, continue testing with the oxidase test. Oxidase testing methods are presented in Chapter V “*Neisseria gonorrhoeae*: Confirmatory Identification and Antimicrobial Susceptibility Testing.”

Confirming pure culture from the primary isolation plate

It is useful to **re-incubate the primary isolation plate and GC-chocolate agar subculture plates for 24 hours after the selection of colonies resembling *N. gonorrhoeae* to determine if colonies of contaminating organisms are present** and were not visible after the first 24 hours. Colonies of staphylococci (gram-positive, oxidase-negative cocci), for example, may be somewhat translucent after incubation for 24 hours, whereas they will form readily distinguishable white, opaque colonies after incubation for 48 hours. Colonies of streptococci (gram-positive, oxidase-negative cocci that often appear as diplococci) may also grow in specimens for gonococci: streptococcal colonies will be very small after incubation for 24 hours but should be clearly visible after incubation for 48 hours and may be surrounded by a zone of α -hemolysis.

Recognition of pure colonies of *N. gonorrhoeae* is often easier after incubation at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 48 hours. Colonies may double in size between 24 and 48 hours, making typical colony characteristics more readily apparent. Repeat the Gram stain (or simple Loeffler’s methylene blue stain) and an oxidase test to confirm that the isolate is an oxidase-positive, gram-negative diplococcus with the typical kidney bean morphology; if the culture is not pure, colonies with morphology typical of gonococcus should be re-streaked over a small section of the primary isolation plate, and incubate the plate at 35°–36.5°C in a CO₂-enriched, humid atmosphere for 24 hours, as described in the primary isolation portion of this chapter. Once the culture is confirmed to be pure *N. gonorrhoeae*, continue with confirmatory identification and antimicrobial susceptibility testing (Chapter VI), and/or preservation and storage of the isolate for future use (Appendix 11). Isolates should always be confirmed as pure prior to storage.

Fecal Specimens: Collection, Transport, and Field Supplies

The information in this appendix is provided to the laboratorian to help ensure appropriate collection of samples and subsequent transport to the laboratory by individuals in the field.

During an outbreak, stool specimens or rectal swabs should be collected from 10–20 persons who meet the following criteria:

- currently have watery diarrhea (cholera) or bloody diarrhea (dysentery)
- had onset of illness <4 days before sampling; and,
- have not received antimicrobial treatment for the diarrheal illness.

Fecal specimens should be collected in the early stages of any enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started (Table 31). An exception to this rule is when stool is collected from persons with febrile illness: in the case of typhoid fever, the etiologic agent *Salmonella* ser. Typhi may be present in highest numbers in stool in the second and third weeks of the disease.

Collection of stool

Stools samples should be collected in clean containers without disinfectant or detergent residue and with tight-fitting, leak-proof lids. Specimens should **not** be collected from bedpans, because the bedpans may contain residual disinfectant or other contaminants. **Unpreserved stool should be refrigerated, if possible, and processed within a maximum of 2 hours after collection. Specimens that cannot be cultured within 2 hours of collection should be placed in transport medium and refrigerated immediately.**

Transport media for fecal specimens

This section provides information regarding media appropriate for the transport of fecal specimens that are suspected to contain *Shigella*, *Vibrio cholerae*, or *Salmonella* (including serotype Typhi) specimens. Once specimens from an outbreak of diarrheal disease have arrived at the laboratory, laboratorians should

TABLE 31: Collection and transport of fecal specimens for laboratory diagnosis

Fecal Specimens for the Laboratory	When to collect	When the patient is having diarrhea, as soon after onset of illness as possible (preferably within 4 days of onset) and before antimicrobial treatment is started.
	How much to collect	Rectal swab or swab of fresh stool in transport medium.
	Transport medium	Cary-Blair or other suitable transport medium (NOT buffered glycerol saline for <i>V. cholerae</i>).
	Storage after collection	Refrigerate at 4 °C if the specimens will be received by the laboratory within 48 hours or freeze at -70 °C. Fecal specimens from patients with suspected cholera can be transported at ambient temperature and held for longer times if necessary; however, refrigeration is preferred.
	Transportation	Seal tubes/containers to prevent leakage; place in waterproof container to protect from wet or dry ice. Ship in insulated box with ice packs, wet ice, or dry ice by overnight delivery.

follow procedures for *Shigella* or *V. cholerae* isolation (Appendix 10) depending on whether reports from the field indicate the outbreak appears to be dysentery or a cholera-like illness. Because persons suspected of having typhoid will commonly present with fever and not diarrhea, laboratories usually do not receive a surge of fecal specimens in an outbreak of typhoid; however, on occasion fecal specimens may be submitted to a laboratory for diagnosis of infection with *S. Typhi* (see Appendix 10 for isolation methods).

Cary-Blair transport medium

Cary-Blair transport medium can be used to transport many bacterial enteric pathogens, including *Shigella*, *Salmonella*, and *Vibrio cholerae* (Figure 81). Cary-Blair’s semisolid consistency provides for ease of transport, and the prepared medium can be stored after preparation at room temperature for up to 1 year. Because of its high pH (8.4), it is the medium of choice for transport and preservation of *V. cholerae*.

Other transport media

Other transport media that are similar to Cary-Blair are Amies’ and Stuart’s transport media. Both of these are acceptable for *Shigella* and *Salmonella* (including ser. Typhi), but they are inferior to Cary-Blair for transport of *V. cholerae*.

Alkaline peptone water may be used to transport *V. cholerae*, but this medium is inferior to Cary-Blair and should be used only when the latter medium is not available. **Alkaline peptone water should not be used if subculture will be delayed more than 6 hours from the time of collection**, because other organisms will overgrow vibrios after 6 hours.

Buffered glycerol saline (BGS), a transport medium that is used for *Shigella*, is unsuitable for transport of *V. cholerae*. Additional disadvantages of buffered glycerol saline are that it can be used for only 1 month after it is made and, because it is a liquid medium, it is more likely to leak or spill during transport.

FIGURE 81: Cary-Blair semisolid transport medium



Placing stool in transport medium

If possible, chill the transport medium for 1–2 hours in a refrigerator or cold box prior to use. A small amount of stool can be collected by inserting a sterile cotton- or polyester-tipped swab into the stool and rotating it. If mucus and shreds of intestinal epithelium are present, these should be sampled with the swab. Following sampling of the stool on the swab:

- a) Insert the swab containing fecal material into transport medium immediately.
- b) Push the swab completely to the bottom of the tube of transport medium.
- c) Break off the top portion of the stick touching the fingers and discard it.
- d) Replace the screw cap on the tube of transport medium and tighten firmly.
- e) Place the tube in a refrigerator or cold box.

Collection of rectal swabs

Sometimes rectal swabs are collected instead of stool specimens. Rectal swabs may be collected as follows:

- a) Moisten the swab in sterile transport medium.
- b) Insert the swab through the rectal sphincter 2–3 cm (*i.e.*, 1–1.5 inches) and rotate.
- c) Withdraw the swab from the rectal sphincter and examine to make sure there is some fecal material visible on the swab. (If not, repeat the procedure with the same swab.)

- d) Immediately insert the swab into cold transport medium (as described in the preceding section).
- e) Place the tube in a refrigerator or cold box.

The number of swabs needed will depend on the number of plates to be inoculated. **In general, if specimens will be examined for more than one pathogen, at least two stool swabs or rectal swabs should be collected per patient, and both swabs should be inserted into the same tube of transport medium.** Once the swab is placed in the medium, it should remain in the tube until it is processed in the laboratory.

Storage of specimens in transport medium

If transport medium has been stored at room temperature, it should be chilled in a refrigerator or cold-box, if possible, for 1–2 hours before use. Specimens preserved in transport medium should be refrigerated until processed. If specimens will be kept more than 2–3 days before being cultured, it is preferable to freeze them immediately at -70°C . It may be possible to recover pathogens from refrigerated specimens up to 7 days after collection; however, the yield decreases after the first 1 or 2 days. Prompt plating, refrigeration, or freezing of specimens in Cary-Blair is particularly important for isolation of *Shigella*, which is more fragile than other enteric organisms. Fecal specimens in transport medium collected from patients with cholera need not be refrigerated unless they are likely to be exposed to elevated temperatures (*i.e.*, $>40^{\circ}\text{C}$).

Unpreserved specimens

When transport medium is not available, one option for specimens suspected to contain *V. cholerae* is to soak a piece of filter paper, gauze, or cotton in liquid stool and place it into a plastic bag. The bag must be tightly sealed so that the specimen will remain moist and not dry out. Adding several drops of sterile saline to the bag may help prevent drying of the specimen. Refrigeration during transport is desirable but not necessary. **This method is not suitable for transport of *Shigella* or *Salmonella* specimens and is less effective than transport medium for preserving *V. cholerae* organisms.**

Preparing specimens for shipment

Specimen tubes should be clearly labeled with the specimen number, and if possible, the patient's name and date of collection. Write the numbers on the

frosted portion of the specimen tube using an indelible marker pen. If the tube does not have a frosted area, write the information on a piece of first-aid tape and affix this firmly on the specimen container. Patient information should be recorded on a data sheet; one copy should be sent with the specimens and another kept by the sender. (A sample data sheet is provided in Figure 82).

If a package is to be shipped by air, the International Air Transport Association (IATA) regulations presented in the *Dangerous Goods Regulations* (DGR) publication must be followed; these regulations (current as of 2002) are summarized in Appendix 12, “Packing and Shipping of Diagnostic Specimens and Infectious Substances.” Even if the package will be shipped by other means, these regulations are excellent guidelines for packing all infectious or potentially infectious materials.

Refrigerated specimens

Refrigerated specimens should be transported to the laboratory in an insulated box with frozen refrigerant packs or ice. If wet ice is used, the tubes or containers should be placed in waterproof containers (*e.g.*, plastic bags) that can be tightly sealed to protect the specimens from the water formed by melting ice.

Frozen specimens

Frozen specimens should be transported on dry ice. The following precautions should be observed:

- Place tubes in containers or wrap them in paper to protect them from dry ice. Direct contact with dry ice can crack glass tubes.
- If the specimens are not in leak-proof containers, protect them from exposure to carbon dioxide by sealing the screwcaps with tape or plastic film or by sealing the tubes in a plastic bag. Carbon dioxide will lower the pH of the transport medium and adversely affect the survival of organisms in the specimen.
- Ensure that the cool box is at least one-third full of dry ice. If the specimens are sent by air and more than 2 kg of dry ice is used, special arrangements may be necessary with the airlines. Airlines accept packages with less than 2 kg of dry ice.
- Address the package clearly, including the sender’s name and telephone number as well as the name and telephone number of the receiving laboratory.
- Write in large letters: EMERGENCY MEDICAL SPECIMENS; CALL ADDRESSEE ON ARRIVAL; HOLD REFRIGERATED (or “FROZEN”, if applicable).
- Be sure that all applicable labels and forms, such as those required by IATA, are correctly fixed to the outside of the package (Appendix 12, Table 36).

Laboratory supplies for outbreaks of diarrheal disease

It is important that local laboratories in a region prone to outbreaks of diarrheal disease have supplies available to work with in the event of an epidemic. Laboratories at the regional level have different requirements for supplies than either regional or national reference laboratories.

Tables 32 and 33 present lists of supplies for testing of specimens and identification of isolates from suspected outbreaks of dysentery and cholera, respectively. The supply lists provided permit the collection and transport of 50 specimens by the district laboratory, the processing of 100 specimens by the regional laboratory, and the identification (and antimicrobial susceptibility testing, if appropriate) of 500 isolates by the national or central reference laboratory.

Further information regarding the role of the laboratory in epidemics of dysentery and cholera can be found in the World Health Organization-endorsed Centers for Disease Control manual, *Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera*, which was published in 1999; the manual is currently available in English and French. Another useful source of information is the World Health Organization's 1997 publication, *Epidemic Diarrhoeal Disease Preparedness and Response: Training and Practice—Participant's manual*.

TABLE 32: Materials needed to collect, transport, and test specimens from dysentery outbreaks for laboratories at the district level, the regional level, and the national (central) reference level

Supplies	District-level laboratory (based on the collection of 50 specimens from dysentery outbreaks)	Regional-level laboratory (based on the processing of 100 specimens from dysentery outbreaks)	National (or central) reference laboratory (based on the of confirmation 500 isolates of <i>Shigella</i>)
Sterile cotton or polyester swabs	At least 100 swabs	At least 200 swabs	At least 1000 swabs
Cary-Blair (or other transport medium)	50 bottles or tubes	500 grams (100 bottles)	5 x 500 grams
Materials and transportation (to send specimens to higher-level laboratory for additional testing)	(For safe and proper transport to regional laboratory.)	(For safe and proper transport to national laboratory.)	(For safe and proper transport to international reference laboratory.)
Xylose lysine desoxycholate (XLD) medium	~	500 grams	5 x 500 grams
MacConkey medium	~	500 grams	5 x 500 grams
Kligler iron agar	~	500 grams	3 x 500 grams
Motility agar	~	500 grams	3 x 500 grams
Nonselective agar (e.g., tryptone soy agar [TSA] or heart infusion agar [HIA])	~	500 grams	3 x 500 grams
Monovalent <i>S. dysenteriae</i> 1 diagnostic antiserum (Note: not Group A polyvalent)	~	4 x 2-ml bottles	20 x 2-ml
Polyvalent <i>S. flexneri</i> (Group B) diagnostic antiserum	~	2 x 2-ml bottles	10 x 2-ml
Polyvalent <i>S. sonnei</i> (Group D) diagnostic antiserum	~	2-ml bottle	5 x 2-ml
Glass slides for serologic testing	~	At least 300 slides	At least 1500 slides
Disposable Petri plates (9 cm)	~	500 plates	5 x 500 plates
Disposable test tubes (e.g., 13 x 100 mm, or 16 x 125 mm)	~	1000 test tubes	5 x 1000 test tubes
Materials and postage (for the production and dissemination of reports)	~	(Required)	(Required)

TABLE 32: Dysentery supplies, *continued*

Supplies	District-level laboratory (based on the collection of 50 specimens from dysentery outbreaks)	Regional-level laboratory (based on the processing of 100 specimens from dysentery outbreaks)	National (or central) reference laboratory (based on the of confirmation 500 isolates of <i>Shigella</i>)
Antimicrobial susceptibility test supplies for 100 <i>Shigella</i> isolates (for national reference laboratory only)			
• Mueller-Hinton agar	?	?	2 x 500 grams
• Disposable Petri dishes	?	?	200 dishes
• Trimethoprim-sulfamethoxazole [1.25 / 23.75 -µg disks]	?	?	200 disks
• Chloramphenicol [30-µg disks]	?	?	200 disks
• Ampicillin [10-µg disks]	?	?	200 disks
• Nalidixic acid [30-µg disks]	?	?	200 disks
• Ciprofloxacin [5-µg disks]	?	?	100 disks
• NCCLS control strain <i>E. coli</i> ATCC 25922	?	?	(Required)
• 0.5 McFarland turbidity standard	?	?	(Required)
• Sterile cotton swabs	?	?	200 swabs
• Sterile saline	?	?	(Required)
• Forceps	?	?	(Required)
• 95% alcohol for flaming	?	?	(Required)
• Galipers (or ruler on a stick)	?	?	(Required)
• Inhibition zone diameter criteria chart <i>[for interpretation per NCCLS]</i>	?	?	(Required)

TABLE 33: Materials needed to collect, transport, and test specimens from cholera outbreaks for laboratories at the district level, the regional level, and the national (central) reference level

Supplies	District-level laboratory (based on the collection of 50 specimens from cholera outbreaks)	Regional-level laboratory (based on the processing of 100 specimens from cholera outbreaks)	National (or central) reference laboratory (based on the of confirmation 500 isolates of <i>Vibrio cholerae</i>)
Sterile cotton or polyester swabs	At least 100 swabs	At least 200 swabs	At least 1000 swabs
Cary-Blair (or other transport medium)	50 bottles or tubes	500 grams (100 bottles)	5 x 500 grams
Materials and transportation (to send specimens to a higher-level laboratory for additional testing)	(For safe and proper transport to regional laboratory.)	(For safe and proper transport to national laboratory.)	(For safe and proper transport to international reference laboratory.)
Thiosulfate citrate bile salts sucrose (TCBS) agar medium	~	500 grams	5 x 500 grams
Sodium desoxycholate (bile salts)	~	25 grams	5 x 25 grams
Glass slides for string test and serologic testing	~	At least 300 slides	At least 1500 slides
Kovac's oxidase reagent	~	5 grams	5 x 5 grams
Filter paper (for oxidase test)	~	(Required)	(Required)
Sterile wooden sticks or platinum inoculating loops for oxidase test	~	(Required)	(Required)
Nonselective agar* (e.g., tryptone soy agar [TSA] or heart infusion agar) (*do not use nutrient agar, lacking salt)	~	500 grams	5 x 500 grams
Polyvalent <i>V. cholerae</i> O1 diagnostic antiserum	~	4 x 2-ml bottles	20 x 2-ml bottles
<i>V. cholerae</i> O139 diagnostic antiserum	~	~	5 x 2-ml bottles
<i>V. cholerae</i> O1 serotype Ogawa diagnostic antiserum	~	~	5 x 2-ml bottles
<i>V. cholerae</i> O1 serotype Inaba diagnostic antiserum	~	~	5 x 2-ml bottles
Peptone medium for alkaline peptone water (e.g., Bacto-peptone)	~	500 grams	5 x 500 grams
NaCl (note: if using table salt for NaCl, it must not be iodized)	~	500 grams	5 x 500 grams

TABLE 33: Cholera supplies, *continued*

Supplies	District-level laboratory (based on the collection of 50 specimens from cholera outbreaks)	Regional-level laboratory (based on the processing of 100 specimens from cholera outbreaks)	National (or central) reference laboratory (based on the of confirmation 500 isolates of <i>Vibrio cholerae</i>)
NaOH	?	(Required)	(Required)
pH paper or pH meter	?	(Required)	(Required)
Petri plates (9-cm)	?	500 plates	5 x 500 plates
Test tubes (e.g., 13 x 100-mm or 16 x 125-mm)	?	1 000 tubes	5 x 1 000 tubes
Materials and postage (for the production & dissemination of reports)	?	(Required)	(Required)
Antimicrobial susceptibility test supplies for 100 <i>V. cholerae</i> isolates (for national reference laboratory only)			
• Mueller-Hinton agar	?	?	2 x 500 grams
• Disposable Petri plates	?	?	200 plates
• Trimethoprim-sulfamethoxazole [1.25 / 23.75 -µg disks]	?	?	200 disks
• Tetracycline [30-µg disks]	?	?	200 disks
• Nalidixic acid [30-µg disks]	?	?	200 disks
• Ciprofloxacin [5-µg disks]	?	?	100 disks
• NCCLS control strain <i>E. coli</i> / ATCC 25922	?	?	(Required)
• 0.5 McFarland turbidity standard	?	?	(Required)
• Sterile cotton swabs	?	?	200 swabs
• Sterile saline	?	?	(Required)
• Forceps	?	?	(Required)

TABLE 33: Cholera supplies, *continued*

Supplies	District-level laboratory (based on the collection of 50 specimens from cholera outbreaks)	Regional-level laboratory (based on the processing of 100 specimens from cholera outbreaks)	National (or central) reference laboratory (based on the of confirmation 500 isolates of <i>Vibrio cholerae</i>)
<ul style="list-style-type: none"> 95% alcohol for flaming 	~	~	(Required)
<ul style="list-style-type: none"> Calipers (or ruler on a stick) 	~	~	(Required)
<ul style="list-style-type: none"> Inhibition zone diameter criteria chart <i>[for interpretation per NCCLS]</i> 	~	~	(Required)