

Laboratory Processing of Fecal Specimens

This laboratory manual includes three pathogens that may be isolated from fecal specimens: *Shigella*, *Vibrio cholerae* O1/O139, and *Salmonella* serotype Typhi. Methods for the laboratory detection of other enteric pathogens can be found in other manuals, such as the American Society for Clinical Microbiology's *Manual of Clinical Microbiology* or the World Health Organization's *Manual for the Laboratory Investigations of Acute Enteric Infections*. The methods presented in this manual are intended to be economical and to offer laboratorians some flexibility in choice of protocol and media. Laboratories that do not have sufficient resources to adopt the methods described in this chapter should consider sending specimens or isolates to other laboratory facilities that routinely perform these procedures.

Enteric pathogens of public health concern cause both diarrheal disease and fever of unknown origin. Only a few pathogens cause epidemic diarrhea, although many cause sporadic diarrhea. *S. dysenteriae* serotype 1 and *V. cholerae* are the two etiologic agents responsible for most epidemic diarrhea in the developing world, contributing substantially to the burden of morbidity and mortality. *S. Typhi*, the etiologic agent of typhoid fever, is responsible for a substantial portion of the burden of fever of unknown origin.

In countries at risk for epidemics of dysentery or cholera, the laboratory's first role is to be prepared for an epidemic; this means having ready access to the supplies necessary to identify *V. cholerae* O1/O139 and *Shigella*. Appendix 9 lists laboratory supplies required for isolation, identification, and antimicrobial susceptibility testing, as appropriate for district-level laboratories, regional laboratories, and national reference laboratories. All countries should have at least one national or central laboratory capable of identifying *Shigella* and *V. cholerae* O1/O139, determining antimicrobial susceptibility, and sending isolates to a regional or international reference laboratory; Appendix 12 includes international shipping regulations and Appendix 14 lists international reference laboratory contact information.

Collection, storage, and transport of stool specimens are addressed in Appendix 9. Methods for isolation of *S. Typhi*, *V. cholerae*, and *Shigella* from stool specimens are detailed in this appendix, whereas each of the pathogen-specific chapters address pathogen identification and antimicrobial susceptibility testing methods, including guidelines for interpretation of results to help shape patient treatment and policy.

Serogrouping and typing methodologies are included and these procedures are encouraged, when resource levels at the laboratory permit.

(*S. Typhi* is included in Chapter VII; *Shigella* is included in Chapter VIII; and, *V. cholerae* is included in Chapter IX.)

Determination of antimicrobial susceptibility patterns not only helps shape successful treatment plans for individual patients but also assists with the development of public health policy for populations at risk for exposure. As mentioned in the introduction to this laboratory manual, because antimicrobial susceptibility testing is so resource intensive and requires a consistent investment in laboratory infrastructure and quality control, the World Health Organization (WHO) recommends that antimicrobial susceptibility testing occur at only one or two laboratories in a country with limited resources. Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. Peripheral laboratories may perform initial isolation of *Salmonella* (including serotype Typhi), *Vibrio*, and *Shigella* isolates, and then refer isolates to the central or national reference laboratory for final confirmation and determination of antimicrobial susceptibility. Peripheral laboratories may also be the sites of focused studies to determine etiologic agents causing an outbreak. First-level laboratories should be supplied with transport medium and the means of sending the specimens to the next level laboratory or to the central laboratory.

Fecal specimens in the laboratory

Once specimens have arrived at the laboratory, laboratorians should follow procedures to isolate the suspected etiologic agent. In an outbreak situation, usually either dysentery or cholera is suspected on the basis of reports from health personnel in the field, and the laboratory response should reflect this. It should be noted that although some health-care providers believe that diarrheal illnesses can be diagnosed by the appearance of the stool and, for example, diagnose dysentery if the stool is bloody and cholera if the stool is watery, this “bloody” versus “watery” distinction is by no means definitive. Diarrhea caused by *Shigella*, for example, is only bloody approximately 50% of the time, and there are many agents that lead to watery diarrhea. Still, clinical observations may help guide laboratory testing.

Laboratories may also receive fecal (*i.e.*, stool) specimens from patients who are suspected to have typhoid fever. Fecal cultures may be positive during the first week of fever and may be positive 2–3 weeks into the disease. (Because *S. Typhi* is more commonly suspected in cases of febrile illness and isolated from blood, urine, or bone marrow, pertinent isolation techniques are also included in Appendix 4, “Isolation of Agents from Normally Sterile Sites.”)

Recovery of *S. Typhi* from fecal specimens

Maximal recovery of *Salmonella* ser. Typhi from fecal specimens is obtained by using an enrichment broth although isolation from acutely ill persons may be possible by direct plating. Enrichment broths for *Salmonella* are usually highly selective and will inhibit certain serotypes of *Salmonella* (particularly *S. Typhi*). The selective enrichment medium most widely used to isolate *S. Typhi* from fecal specimens is selenite broth (SEL). Selenite broth should be incubated for 14–16 hours at 35°–37°C and then streaked to selective agar (e.g., bismuth sulfite [BS] or desoxycholate citrate agar [DCA]). A nonselective broth (e.g., Gram negative [GN] broth) may also be used for enrichment for *S. Typhi*.

Plating media

Fecal specimens to be examined for *S. Typhi* may be inoculated onto standard enteric plating media (e.g., Hektoen enteric agar [HE], xylose lysine desoxycholate agar [XLD], DCA, MacConkey agar [MAC], or *Salmonella-Shigella* [SS] agar). However, bismuth sulfite agar (BS) is the preferred medium for isolation of *S. Typhi* and should be used if resources permit.

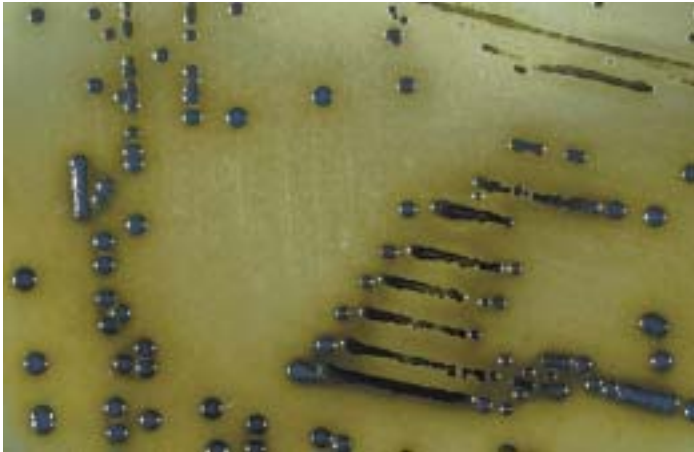
BS plates must be freshly prepared (Appendix 2) and used within 36 hours for isolation of *S. Typhi*. A rectal swab or stool swab may be used to inoculate BS agar by seeding an area approximately 1 inch in diameter on the agar, after which the plate is streaked for isolation. After seeding the plate, the swab may be placed in a tube of selenite broth if enrichment is desired.

If culturing fecal specimens from suspected typhoid carriers, the use of a BS pour plate may enhance isolation. For pour plates, the BS agar must be boiled and cooled to 50°C in a water bath. A 5-ml quantity of fecal suspension is added to a Petri plate, after which approximately 20 ml of cooled BS is immediately poured into the plate. The plate is swirled to mix the fecal suspension and the BS agar and the plate is left to harden.

BS streak and BS pour plates should be incubated for 48 hours at 35°–37°C. On a BS streak plate, well-isolated colonies of *S. Typhi* appear black surrounded by a black or brownish-black zone with a metallic sheen. On a BS pour plate, well-isolated subsurface colonies are black and circular. Table 34 provides descriptions of *S. Typhi* colonies on other types of selective media. When colonies of *S. Typhi* are numerous and crowded, *S. Typhi* frequently does not produce typical blackening of BS; therefore, **plates must be streaked carefully to permit growth of discrete colonies**. When using pour plates, a second plate with a 0.5-ml inoculum may also be prepared to insure that isolated colonies will develop. Figure 83 illustrates the appearance of *S. Typhi* colonies on BS agar medium.

A flowchart for the isolation and identification of *S. Typhi* is included in Figure 29. Isolated colonies from BS or other selective media may be inoculated to Kligler iron agar (KIA) or triple sugar iron agar (TSI) or other screening media.

FIGURE 83: *Salmonella* ser. Typhi colonies on bismuth sulfite (BS) agar



Sub-surface colonies from BS pour plates must be re-streaked for isolation on a medium such as MAC before being inoculated into KIA or TSI.

Colonies of *S. Paratyphi* A, *S. Paratyphi* B, and *S. Paratyphi* C and most other *Salmonella* serotypes have a similar appearance to *S. Typhi* on MAC, BS, HE, DCA, and XLD agar. Methodology for confirmatory identification and antimicrobial susceptibility testing of *S. Typhi* is addressed in Chapter VII.

TABLE 34: Appearance of *Salmonella* ser. Typhi colonies on selective plating media

Selective agar medium*	Color of colonies*	Size of colonies*	Figure number
Bismuth sulfite agar (BS)	Black, surrounded by a black or brownish zone with a metallic sheen	1 – 3 mm	Figure 83
MacConkey agar (MAC)	Transparent or colorless opaque	2 – 3 mm	Figure 59a
Hektoen enteric agar (HE)	Blue-green (with or without black centers) or yellow with black centers	1 – 2 mm	~
Xylose lysine desoxycholate agar (XLD)	Red (with or without black centers) or yellow with black centers	1 – 2 mm	~
<i>Salmonella-Shigella</i> (SS) agar	Colorless	1 – 2 mm	~
Desoxycholate citrate agar (DCA)	Colorless	1 – 2 mm	~

* Most *Salmonella* serotypes appear similar to *S. Typhi* on these media; therefore, confirmatory testing is necessary.

Recovery of *Shigella* from stool: Isolation and preliminary identification

Isolation and identification of *Shigella* can be greatly enhanced when optimal laboratory media and techniques are employed.

An outline of the procedure for isolation and identification of *Shigella* from fecal specimens is presented in Figure 36. Refer to Appendix 9 for a list of supplies necessary for laboratory identification of *Shigella*. (This appendix includes supplies appropriate for district laboratories, regional laboratories and national reference laboratories.) A sample worksheet for organizing laboratory data is presented in Figure 37.

There is no enrichment medium for *Shigella* that consistently provides a greater recovery rate than use of direct plating alone. For optimal isolation of *Shigella*, two different selective media should be used: a general purpose plating medium of low selectivity, such as MAC, and a more selective agar medium, such as XLD. DCA and HE agar are suitable alternatives to XLD agar as media of moderate to high selectivity. **SS agar should not be used** because it frequently inhibits the growth of *S. dysenteriae* serotype 1.

Inoculation of selective agar for recovery of *Shigella* from fecal specimens

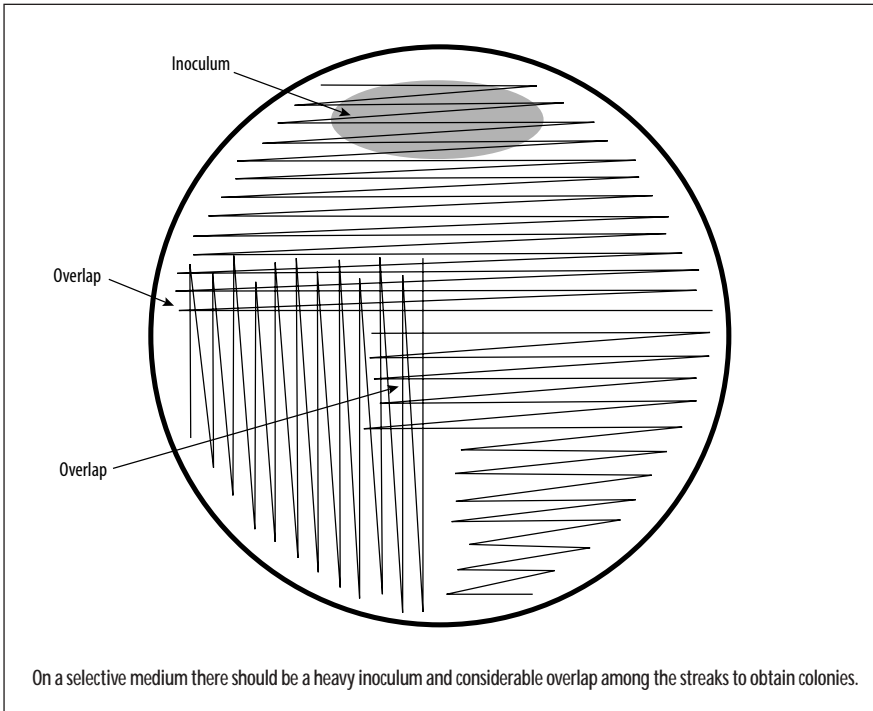
Fecal specimens should be plated as soon as possible after arrival in the laboratory. Selective media may be inoculated with a single drop of liquid stool or fecal suspension. Alternatively, a rectal swab or a fecal swab may be used. If a swab is used to inoculate selective media, an area approximately 2.5 cm (1 inch) in diameter is seeded on the agar plates, and the plates then are streaked for isolation (Figure 84).

When inoculating specimens to a plate for isolation, the entire surface of the agar plate must be used to increase the chances of obtaining well-isolated colonies. Media of high selectivity (e.g., XLD) require more overlapping when streaking than media of low selectivity (e.g., MAC); it is therefore important to pay particular attention to streaking. After streaking, cover the agar plate and place it upside-down (i.e., cover-side down) in the incubator to avoid excessive condensation. Incubate the plates for 18–24 hours at 35°–37°C.

Isolation of suspected *Shigella* from selective media

After incubation, record the amount and type of growth (i.e., lactose-fermenting or -nonfermenting) on each isolation medium for each patient specimen. Colonies of *Shigella* on MAC appear as convex, colorless colonies approximately 2–3 mm in diameter, although *S. dysenteriae* 1 colonies may be smaller (Table 35). *Shigella* colonies on XLD agar are transparent pink or red, smooth colonies, approximately 1–2 mm in diameter, although *S. dysenteriae* 1 colonies on XLD agar are frequently

FIGURE 84. Method of streaking plating medium for isolation of *Shigella* and *Vibrio* species



very tiny. Select suspect colonies from the MAC and XLD plates and inoculate them to appropriate screening media such as Kligler iron agar (KIA) or triple sugar iron agar (TSI). Figures 85, 86, 87, and 88 show the typical appearance of *Shigella* colonies on XLD and MAC.

Following the preliminary identification of suspect *Shigella* colonies on plating media, the laboratorian should conduct biochemical screening tests and serologic testing to confirm the identification of the agent. Methodology for the identification and antimicrobial susceptibility testing of *Shigella* is addressed in Chapter VIII of this manual.

Recovery of *V. cholerae* from stool: Isolation and preliminary identification

Although *V. cholerae* will grow on a variety of commonly used agar media, isolation from fecal specimens is more easily accomplished with specialized media. Alkaline peptone water is recommended as an enrichment broth, and thiosulfate citrate bile salts sucrose agar (TCBS) is the selective agar medium of choice. (Refer to Appendix 2 (“Media, Reagents and Quality Control”) before preparing any of these media because incorrect preparation can affect the reactions of organisms in

TABLE 35: Appearance of *Shigella* colonies on selective plating media

Selective agar medium	Color of colonies	Size of colonies	Figure number
MacConkey agar (MAC)	Colorless	2 – 3 mm ^{a,b}	Figure 88
Xylose lysine desoxycholate (XLD)	Red or colorless	1 – 2 mm ^{a,c}	Figures 85, 86, and 87
Desoxycholate citrate agar (DCA)	Colorless	2 – 3 mm ^a	~
Hektoen enteric agar (HE)	Green	2 – 3 mm ^a	~

^a *S. dysenteriae* 1 colonies may be smaller.

^b See Appendix 2 for discussion of different formulations of commercial dehydrated MacConkey agar and how selectivity is affected for isolation of *Shigella*.

^c *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species.

these tests.) Figure 45 provides a schematic representation for the recovery and identification of *V. cholerae* from fecal specimens.

Enrichment of suspected *V. cholerae* in alkaline peptone water

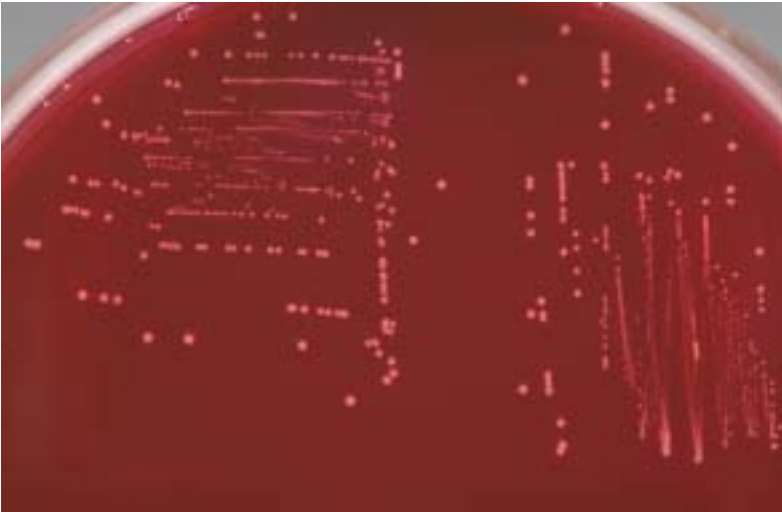
Enrichment in alkaline peptone water (APW) can enhance the isolation of *V. cholerae* when few organisms are present, as in specimens from convalescent

FIGURE 85: *Shigella dysenteriae* 1 colonies on xylose lysine desoxycholate (XLD) agar



The colonies appear as small pinpoints of growth; this pattern is characteristic of growth of *S. dysenteriae* type 1 on XLD specifically, and can help guide in the identification of the etiologic agent.

FIGURE 86: *Shigella flexneri* colonies on xylose lysine desoxycholate (XLD) agar



Colonies of *S. flexneri* are larger on XLD than are colonies of *S. dysenteriae* 1.

FIGURE 87: *Shigella flexneri* and *Escherichia coli* colonies on xylose lysine desoxycholate (XLD) agar



S. flexneri colonies are colorless to red, whereas *E. coli* colonies are yellow on XLD.

FIGURE 88: *Shigella flexneri* and *Escherichia coli* colonies on MacConkey (MAC) agar



S. flexneri colonies appear colorless on MAC, whereas *E. coli* colonies are pink to red.

patients and asymptomatic carriers. *Vibrio* spp. grow very rapidly in alkaline peptone water, and at 6–8 hours they will be present in greater numbers than non-*Vibrio* organisms.

Alkaline peptone water can be inoculated with liquid stool, fecal suspension, or a rectal swab. The stool inoculum should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35°–37°C for 6–8 hours. After incubation, subculture one to two loopfuls of alkaline peptone water to thiosulfate citrate bile salts sucrose (TCBS) medium. (The loopfuls of APW should be obtained from the surface and topmost portion of the broth, because vibrios preferentially grow in this area.) **Do not shake or mix the tube before subculturing.** If the broth cannot be plated after 6–8 hours of incubation, subculture a loopful of the broth at 18 hours to a fresh tube of alkaline peptone water; this second tube of APW should then be subcultured to TCBS agar after 6–8 hours of incubation.

Inoculation and isolation of suspected *V. cholerae* from thiosulfate citrate bile salts sucrose (TCBS) selective agar

TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective. **Growth from TCBS medium is not suitable for direct testing with *V. cholerae* antisera.**

Inoculate the TCBS plate by streaking (as described in Figure 84). After 18–24 hours' incubation at 35°–37°C, the amount and type of growth (*i.e.*, sucrose-fermenting or sucrose-nonfermenting) on the TCBS plate should be recorded on data sheets (Figure 46). **Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2–4 mm in diameter** (Figure 89). The yellow color is caused by the fermentation of sucrose in the medium; in contrast, sucrose-nonfermenting organisms (*e.g.*, *V. parahaemolyticus*) produce green to blue-green colonies.

Isolation of suspected V. cholerae

Carefully select at least one of each type of sucrose-fermenting (yellow) colony from the TCBS plate to inoculate a heart infusion agar (HIA) slant or another nonselective medium; each type of colony selected should be inoculated onto a separate plate. (*V. cholerae* requires 0.5% NaCl [salt] for optimal growth on agar media; some commercially available formulations of nutrient agar do not contain salt, and should not be used for culture of *V. cholerae*.) Using an inoculating needle, lightly touch only the very center of the colony. (Do not take the whole colony or go through the colony and touch the surface of the plate because contaminants may be on the surface of the agar.) If there is doubt that a particular

FIGURE 89: Growth of *Vibrio cholerae* on thiosulfate citrate bile salts sucrose (TCBS) agar



Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2–4 mm in diameter. The yellow color is caused by the fermentation of sucrose by the organism; non-sucrose-fermenting organisms (*e.g.*, *V. parahemolyticus*) produce green to blue-green colonies on this same medium.

colony is sufficiently isolated from surrounding colonies, purify the suspicious colony by streaking on another agar plate, incubating it and then testing colonies from the subculture.

Incubate the heart infusion agar slants at 35°–37°C for up to 24 hours; note that sufficient growth for serologic testing might be obtainable after 6 hours. Slide serology with polyvalent O1 and O139 antisera is sufficient for a presumptive identification of *V. cholerae*, and is described in Chapter IX of this manual.

Following the preliminary identification of suspect colonies as *V. cholerae* on TCBS agar, the laboratorian should conduct other biochemical and serologic identification tests and, if appropriate, antimicrobial susceptibility testing of the isolate. Methodology for the identification and antimicrobial susceptibility testing of *V. cholerae* is addressed in Chapter IX.

Preservation and Storage of Isolates

It is often necessary for isolates to be examined at a time-point following the infection from which the culture was obtained. For example, it is sometimes appropriate to refer back to an isolate for epidemiological purposes; *e.g.*, to learn if a new case-patient is infected with the same strain of a pathogen as an individual who had an earlier case of disease. Another example would be a situation where a laboratory chooses to screen a number of isolates at one time each year to additional antimicrobial agents or, *e.g.*, for beta-lactamase production; this practice would assist in the detection of emerging characteristics in known pathogens. Sometimes isolates need to be sent to reference laboratories for confirmation and/or further testing and must be stored prior to packing and shipping (Appendix 12). Selection of a storage method depends on the length of time the organisms are to be held and the laboratory equipment and facilities available.

Short-term storage may be accomplished with transport media, freezing, or, in some cases (and for some pathogens) at room temperature on simple media plus mineral oil to prevent drying. Methods for short-term storage appropriate to the different bacteria included in this laboratory manual are included later in this appendix.

Long-term storage of bacterial isolates is best accomplished by either lyophilization or freezing. Specific methods appropriate for the bacteria included in this laboratory manual are included later in this appendix. Lyophilization (freeze-drying) is the most convenient method of storage because lyophilized bacteria can be stored for long periods at 4°C or -20°C and can be transported without refrigeration.⁴¹ However, the equipment required is expensive and not all laboratories will have the ability to lyophilize isolates. (Reference laboratories choosing to lyophilize bacteria should always maintain a frozen preparation in addition to larger quantities of lyophilized strains, because some lyophilized preparations may be nonviable upon reconstitution.) Bacterial cultures may be stored frozen or lyophilized in a variety of suspending media formulated for that purpose. There are many formulations of suspending medium, but in general serum-based media, skim milk, or polyvinylpyrrolidone (PVP) medium is used for

⁴¹ Cultures for transport should be packaged according to the IATA shipping regulations presented in Appendix 12. No more than 50-ml of culture should be shipped in one package.

lyophilization, and skim milk, blood, or a rich buffered tryptone soy broth (TSB) with 15%–20% reagent-grade glycerol is used for freezing. **Do not use human blood**, because of safety issues (e.g., HIV and hepatitis transmission), and because of the possible inhibition of growth of isolates resulting from antibodies or residual antibiotics.

Cultures to be prepared for either permanent or short-term storage should be confirmed as pure before proceeding with any of these methodologies. Fresh cultures (i.e., overnight growth) should be used for the preparation of storage strains.

Storage of *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* isolates

The three agents of pneumonia and meningitis included in this laboratory manual (*H. influenzae*, *N. meningitidis*, and *S. pneumoniae*) are fragile and care must be taken in their preparation for storage. Maintain sterility at all times during preparation of cultures for storage.

Short-term storage of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*

If Dorset Egg medium (DE) is available to the laboratory, it is useful for room temperature (i.e., approximately 25°C) storage of *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*. On DE, *H. influenzae* and *N. meningitidis* can each be stored for approximately 3 weeks, whereas *S. pneumoniae* can be stored for approximately 6 weeks on DE. (Instructions for preparation of DE are included in Appendix 2.) Use overnight growth from blood or chocolate agar, as appropriate, to inoculate a 4-ml DE slant in a 7-ml screw-top tube.

If DE is not a medium readily prepared or used by the laboratory, short-term storage of any of these three pathogens can be carried out on supplemented chocolate agar for up to 1 week.

- Viability during the short-term storage (7 days or fewer) is best if *S. pneumoniae* and *H. influenzae* are inoculated onto chocolate agar slants with screw-cap tubes, incubated overnight at 35°C, and then maintained at 4°C. These bacterial species do not survive well in broth and survive only 3 to 4 days on primary agar plates.
- For *N. meningitidis*, solid screw-caps should be loosened during storage but permeable membrane screw caps (which allow for an exchange of gases and are available commercially) should be used when possible. An overlay of TSB may also be helpful and might increase viability to 14 days. ***N. meningitidis* slants should not be refrigerated.**

S. pneumoniae, *H. influenzae*, and *N. meningitidis* can also be stored short-term on swabs stored in silica gel packets; stored in this manner, the isolates will last approximately 2 weeks at room temperature. The packets are inexpensive and easy to use, but are not often available from commercial manufacturers. (One commercial source of silica gel packets is Scientific Device Laboratory, Inc., included in Appendix 13.) Figure 90 shows how to use the packets.

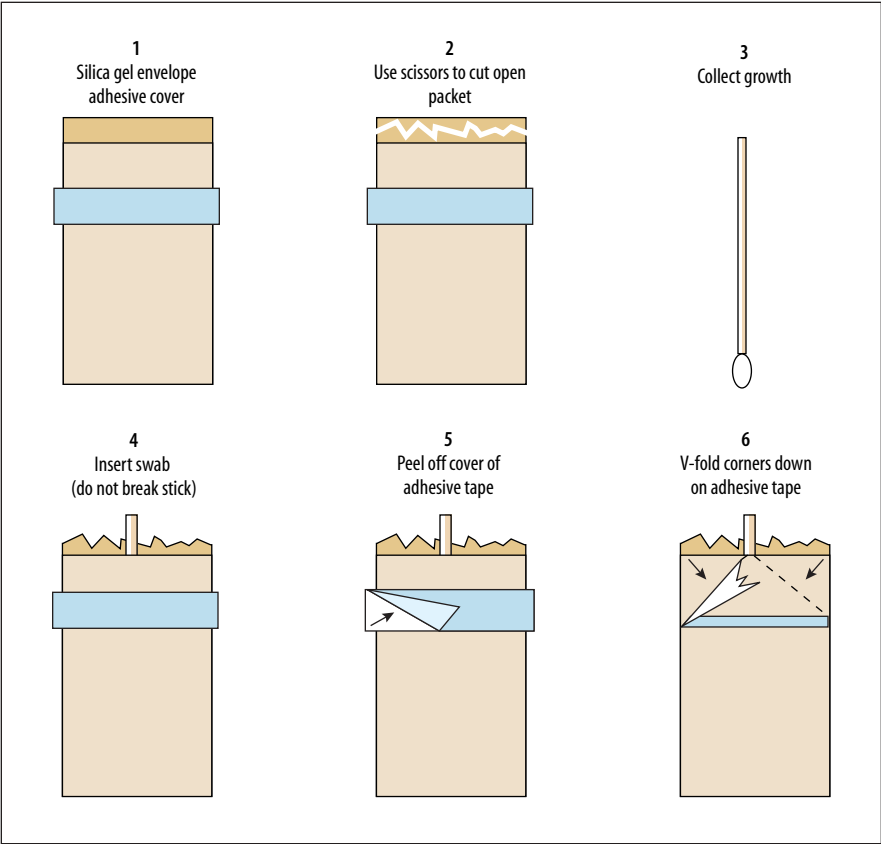
Long-term storage of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*

Long-term storage can be accomplished by freezing or lyophilization.

- **Frozen storage**

- a) Grow pure culture of *H. influenzae* on chocolate agar and of *S. pneumoniae* and *N. meningitidis* on blood or chocolate agar. Incubate the plates in a CO₂ incubator or candle-jar for 18–24 hours at 35°C. Inspect the plates for purity.

FIGURE 90: Silica gel packets for transport (and short-term storage)



- b) Harvest all of the growth from a plate with a sterile swab.
- c) Dispense the growth in a 2-ml, externally-threaded screw-capped cryogenic vial containing 1 ml of sterile defibrinated blood by twirling the swab to release the organisms. Squeeze the excess blood from the swab by rotating it against the sides of the vial before carefully withdrawing it. Discard the swab in disinfectant.
 - Defibrinated sheep, horse, or rabbit blood can be used for all three of these respiratory organisms. **Human blood should not be used.** Alternatives, such as TSB with 15%–20% reagent-grade glycerol or Greaves solution, can also be used.
 - **Caution:** Do not use glass ampoules (*i.e.*, glass cryovials) for freezing in liquid nitrogen because they can explode upon removal from the freezer.
- d) If possible, rapidly freeze the suspension in a bath of 95% alcohol and dry-ice pellets.
- e) Place the cryovials in a -70° freezer or a nitrogen freezer (-120°C). A -20°C freezer can be used, but some loss of viability can be expected. **Freezers with automatic defrosters should never be used.**

- **Lyophilization**

Some laboratories may have lyophilization (*i.e.*, freeze-drying) facilities.

- a) Grow the *H. influenzae* on supplemented chocolate agar / the *S. pneumoniae* and *N. meningitidis* on blood agar or chocolate agar. Incubate the plates in a CO₂-incubator or candle-jar for 18 – 20 hours at 35°C. Inspect the plate for purity.
- b) Harvest the growth from the plate with 1–2 ml of sterile skim milk and a sterile swab. Place approximately 0.5 ml of suspension into a sterile ampoule or lyophilization vial. Several vials can be prepared from a single plate, if desired. **Maintain sterility at all times during the preparation of the vial.**
- c) The cell suspension should be shell-frozen on the walls of the lyophilization vial. This is accomplished by one of the following two methods:
 - Keep the lyophilization vial at -70°C until just before the cell suspension is added. Add the cell suspension and rapidly rotate the vial to freeze the suspension to the wall. Return the vial to the -70°C freezer until ready to attach to the lyophilizer.

or

 - If a -70°C freezer is not available, a mixture of alcohol (95% ethanol) and dry ice can be prepared and used to shell-freeze the cell suspensions. Shell-freezing is accomplished by placing the cell suspension in the lyophilization vial and rotating the vial at a 45° to 60° angle in the alcohol/dry-ice mixture.

- d) Attach the vials to the lyophilizer. **Follow the manufacturer's directions because each instrument uses a different type of apparatus.** The time of lyophilization will depend on the number of vials being lyophilized and the capacity of the instrument. On an average machine, 4–5 hours are required to completely dry 10–20 small vials.
- e) At the end of the run, seal the vials with a torch while they are still attached to the lyophilizer and under vacuum. The vials can be stored at 4°C or at freezer temperatures after being sealed.

- **Recovery of isolates from long-term storage**

Lyophilized specimens of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* can be recovered by suspending the preparation in 0.25–0.5 ml of broth (e.g., TSB, Mueller-Hinton broth, or PBS). Add one drop of the suspension to a plate of medium (sheep blood agar plate or chocolate agar for *H. influenzae*), and approximately five drops to a liquid (broth) medium containing five drops of blood (sheep, rabbit, goat, or horse blood, but **not human blood**). Incubate the plate and tube for 18–24 hours at 35°C, and observe for growth. If growth on the plate occurs, the tube can be discarded; however, if no growth is observed on the plate, sample the medium in the tube and re-incubate. After another 18–24 hours, the plate should be re-examined for growth. If growth is seen, the tube can be discarded; if no growth is present, examine the tube for turbidity (which would indicate growth). If the tube is turbid, the tube should be re-sampled and re-incubated; if the tube is not turbid, assume the lyophilized sample was dead. (This is why it is strongly suggested that a specimen be prepared for long-term frozen storage in addition to lyophilization.) Organisms grown from lyophilized specimens must be subcultured at least once prior to being used in tests.

Frozen cultures should be thawed at room temperature, and a Pasteur pipette should be used to remove a small amount of inoculum from the cryotube for culture. The inoculum may be taken from the frozen culture before the preparation is completed thawed and should be taken no later than when the frozen culture has completely thawed. (Once completely thawed, the frozen culture will begin to lose viability.) Organisms grown from frozen specimens must be subcultured at least once prior to being used in tests.

Storage of *Neisseria gonorrhoeae* isolates

N. gonorrhoeae is a fragile organism and care must be taken in preparation of the cultures for storage. Maintain sterility at all times during preparation of cultures for storage.

Short-term storage of *N. gonorrhoeae*

Isolates of *N. gonorrhoeae* can be stored for approximately 2 weeks at -20°C . (They **cannot be stored at room temperature or 4°C** ; they must be frozen.) Isolates for short-term storage should be stored in TSB containing 20% glycerol at the back of the freezer shelves and not in the door or at the front of the shelves (because when the door to the freezer is opened and the isolates are not at the back of the shelf, they may thaw and not properly refreeze). Repeated freezing/thawing cycles, or failure to re-freeze results in a rapid loss of viability.

Long-term storage of *N. gonorrhoeae*

The best method for storing gonococcal isolates is to freeze them in a -70°C freezer or in liquid nitrogen (at -196°C). Strains may be stored as freeze-dried lyophiles; however, this method is expensive and labor-intensive and lyophiles may lose viability over time.

- **Frozen storage**

To store frozen isolates, use a sterile swab to prepare dense suspensions of 18- to 24-hour pure cultures prepared in TSB containing 20% (vol/vol) glycerin. The best suspensions are prepared by rolling the swab over isolated colonies or the margin of confluent areas of growth. Dispense the suspension into cryovials (*i.e.*, freezing vials specially designed for use at very low temperatures), but **glass ampoules should never be used for freezing in liquid nitrogen** because they can explode upon removal from the freezer.

When frozen suspensions are thawed to inoculate cultures, the suspension should not be refrozen; new suspensions of organisms should be prepared. As many as 99% of the cells in a suspension may be destroyed during the freezing and the thawing of the preparations due to physical destruction (*i.e.*, shearing) of cells by crystals of the suspending medium that form during the freezing processes. One way to minimize the loss of cells during freezing is by “flash-freezing” the specimen in an acetone or alcohol bath containing dry ice. Alternatively, a sample may be taken from the top of the frozen preparation with a sterile bacterial loop if the suspension is not thawed.

If neither a -70°C freezer nor a liquid-nitrogen storage facility is available, gonococcal suspensions may be frozen for **up to 2 weeks at -20°C** ; frozen suspensions of *N. gonorrhoeae* will lose viability if stored for periods longer than 2 weeks at this temperature.

- **Lyophilization**

Some laboratories may have lyophilization (*i.e.*, freeze-drying) facilities. To prepare lyophiles, 18- to 24-hour pure cultures of isolates are suspended in special lyophilization media and are distributed in small aliquots (usually

0.25–0.5 ml) in lyophilization ampoules. As with frozen storage, approximately 99% of the organisms are killed during the freezing process.

Gonococcal isolates should not be suspended in skim milk because fatty acids in the milk may be toxic for some organisms and the density of the suspension cannot be determined. The suspensions are frozen at -70°C or in an ethanol/dry-ice bath and are then dried in a vacuum for 18–24 hours until the moisture has evaporated. **The manufacturer's directions must be followed, because each instrument uses a different type of apparatus.** The dried preparation should be powdery in texture; if the preparation has a clear, syrupy appearance, the vial should be discarded. One ampoule of each strain preparation should also be opened and cultured immediately to ascertain that the preparation is viable and pure and to verify the identity of the organism and its characteristics (*e.g.*, antimicrobial susceptibilities). Ampoules are best stored at $4^{\circ}\text{--}10^{\circ}\text{C}$ or at -20°C ; ampoules should not be stored at room temperature. Oxygen may diffuse slowly into the ampoule through the thin seal, particularly with thin-walled ampoules. Thus, one ampoule should be opened every 1–2 years to confirm that the preparation is viable. If the re-suspended lyophilized preparation does not grow after incubation for 48 hours, new ampoules must be prepared.

- **Recovery of isolates from long-term storage**

Lyophilized specimens of *N. gonorrhoeae* can be recovered by suspending the preparation in 0.5–1.0 ml of glycerol TSB, Mueller-Hinton broth, or PBS, and inoculating GC-chocolate agar. An advantage of using glycerol TSB is that the suspension can be re-frozen until purity is assured on the culture plate; after pure culture is confirmed, the suspension can either be appropriately discarded or a new frozen or lyophilized specimen can be prepared. Perform at least one subculture off the initial culture prior to inoculating tests.

Frozen cultures should be thawed at room temperature, and used to inoculate a plate of GC-chocolate agar. The inoculum may be taken from the frozen culture before the preparation is completely thawed, and should be taken no later than when the frozen culture has completely thawed. (Once completely thawed, the frozen culture will begin to lose viability.)

If resources are available and the stored (lyophilized or frozen) isolate is from a different originating laboratory (*i.e.*, a laboratory other than the one recovering it from the stored specimen), it is suggested that selective GC-medium be inoculated at the same time as GC-chocolate. If the culture is contaminated, this selective medium step will purify the culture.

Storage of *Salmonella*, *Shigella*, and *Vibrio* isolates

Salmonella, *Shigella*, and *Vibrio* isolates will usually remain viable for several days on solid medium held at room temperature (22°–25°C) unless the medium dries out or becomes acidic. However, if cultures are to be maintained for longer than a few days, they should be appropriately prepared for storage. As with other bacteria, selection of a storage method depends on the length of time the organisms are to be held and the laboratory equipment and facilities available. Maintain sterility at all times during preparation of cultures for storage.

Short-term storage of *S. Typhi*, *Shigella*, and *V. cholerae*

Blood agar, tryptone soy agar (TSA), and heart infusion agar (HIA) are examples of good storage media for enteric organisms. Carbohydrate-containing media (e.g., Kligler iron agar [KIA] or triple sugar iron agar [TSI]) should not be used because acidic by-products of metabolism quickly reduce viability of the organisms. Blood agar, TSA, and HIA all contain salt (NaCl), which enhances the growth of *V. cholerae*. (Nutrient agar should not be used for growth or storage of *V. cholerae* because it contains no added salt.)

When preparing storage medium, place tubes of medium that are still hot after autoclaving in a slanted position to provide a short slant and deep butt (2–3 cm). To inoculate, stab the inoculating needle to the butt of the medium once or twice, and then streak the slant. Incubate the culture overnight at 35°–37°C. Seal the tube with cork stoppers that have been soaked in hot paraffin or treated in some other way to provide a tight seal. Store cultures at 22°–25°C and in the dark.

Sterile mineral oil may also be used to prevent drying of slants. Add sufficient sterile mineral oil to cover the slants to 1 cm above the top of the agar, and subculture when needed by scraping growth from the slant; there is no need to remove mineral oil to subculture. ***Shigella*, *Vibrio*, and *Salmonella* strains maintained in pure culture in this manner will usually survive for several years.**

Long-term storage of *S. Typhi*, *Shigella*, and *V. cholerae*

Isolates may be stored indefinitely if they are maintained frozen at -70°C or below; these temperatures can be achieved in an “ultralow freezer” (-70°C) or a liquid nitrogen freezer (-196°C). Storage of isolates at -20°C is not recommended, because some organisms will lose viability at that temperature.

- **Frozen storage**

- a) Inoculate a TSA or HIA slant (or other non-inhibitory, salt-containing growth medium) and incubate at 35°–37°C.
- b) Harvest cells from the slant and make a suspension in the freezing medium.

c) Dispense the suspension into cryovials (freezing vials specially designed for use at very low temperatures).

- **Caution:** Glass ampoules should never be used for freezing in liquid nitrogen because they can explode upon removal from the freezer.

d) Prepare an alcohol and dry-ice bath by placing dry ice (frozen CO₂) in a leak-proof metal container large enough to hold a metal culture rack, and add enough ethyl alcohol to submerge about half of the cryovial. Rapidly freeze the suspension by placing the sealed vials in the dry-ice bath until frozen. (If no dry ice is available, a container of alcohol may be placed in the freezer overnight and then used to quick-freeze vials.) Transfer the frozen vials to the freezer.

- **Lyophilization**

Most organisms may be successfully stored after lyophilization, or freeze-drying. Freeze-drying involves the removal of water from frozen bacterial suspensions by sublimation under reduced pressure. **Follow the manufacturer's directions since each instrument uses a different type of apparatus.** Lyophilized cultures are best maintained at 4°C or lower.

- **Recovery of isolates from long-term storage**

To recover an isolate from frozen storage, remove the frozen cultures from the freezer and place them on dry ice or into an alcohol and dry-ice bath; transfer to a laboratory safety cabinet or a clean area if a cabinet is not available. Using a sterile loop, scrape the top-most portion of the culture and transfer to a growth medium, being careful not to contaminate the top or inside of the vial. Re-close the vial before the contents completely thaw, and return the vial to the freezer; with careful technique, transfers can be successfully made from the same vial several times. Incubate 18–24 hours at 35–37°C; perform at least one subculture before using the isolate to inoculate a test.

To recover lyophilized specimens of *Salmonella*, *Shigella*, or *V. cholerae*, inoculate a tube of nonselective broth (e.g., TSB or heart infusion broth) and incubate the suspension overnight. Subculture the broth to a nonselective growth medium (e.g., TSA or HIA) and incubate 18–24 hours at 35°–37°C.

Packing and Shipping of Diagnostic Specimens and Infectious Substances

Preparation for transport of infectious specimens and cultures

Transport of diagnostic specimens and etiologic agents (*i.e.*, infectious substances) should be done with care not only to minimize the hazard to humans or the environment, but also to protect the viability of suspected pathogens. Transport of infectious items by public or commercial delivery systems may be subject to local, national, and (if crossing national borders) international regulations.

If possible, specimens should be sent so that they will arrive during working hours to ensure proper handling and prompt plating of the specimens. Inform the receiving laboratory as soon as possible that the specimens are coming, preferably before the specimens are sent.

Depending on local conditions, within-country transport may be by ground or by air. If specimens are sent by a messenger, the messenger must know the location of the laboratory and the appropriate person to contact. The sender should identify the fastest and most reliable way of transport in advance (whether it is, *e.g.*, by bicycle, motorcycle, car, ambulance or public transport), and should make sure that adequate funds are available to reimburse costs for fuel or public transport. For longer distances, the fastest transport service may be air-freight or expedited delivery service. Because the ice packs or dry ice will last only 24–48 hours, arrangements should be made for immediate collection at the receiving airport. When the specimens are shipped by air, the following information should be communicated immediately to the receiving laboratory: the air waybill number, the flight number, and the times and dates of departure and arrival of the flight.

Transport and shipment of cultures and specimens

Regulatory organizations

The United Nations Committee of Experts on the Transport of Dangerous Goods is continually developing recommendations for the safe transport of dangerous goods. The International Civil Aviation Organization (ICAO) has used these recommendations as the basis for developing regulations for the safe

transportation of dangerous goods by air. The regulations of the International Air Transport Association (IATA) contain all the requirements of the ICAO Technical Instructions for the Safe Transport of Dangerous Goods. However, IATA has included additional requirements that are more restrictive than those of ICAO. Member airlines of the IATA have adopted the use of the IATA regulations governing dangerous goods, and shippers must comply with these regulations in addition to any applicable regulations of the state of origin, transit, or destination.

The shipment of infectious substances or diagnostic specimens by air must comply with local, national, and international regulations. International air transport regulations may be found in the IATA publication titled *Dangerous Goods Regulations*. This reference is published annually in January and the regulations are often updated each year. A copy of the IATA regulations in English, Spanish, French, or German may be obtained from one of the following regional offices.

Orders for IATA Regulations from the Americas, Europe, Africa, and the Middle East:

Customer Service Representative
International Air Transport Association
800 Place Victoria, P.O. Box 113
Montreal, Quebec
CANADA H4Z 1M1
Telephone: +1 514 390 6726
Fax: +1 514 874 9659
Teletype: YMQTPXB

Orders for IATA Regulations from Asia, Australasia, and the Pacific:

Customer Service Representative
International Air Transport Association
77 Robinson Rd.
No. 05-00 SIA Bldg.
SINGAPORE 068896
Telephone: +65 438 4555
Fax: +65 438 4666
Telex: RS 24200 TMS Ref: TM 2883
Cable: IATAIATA
Teletype: SINPSXB

Internet Information:

<http://www.iata.org>

For Internet Orders, send e-mail to:
sales@iata.org

Shipping regulations for infectious substances and diagnostic specimens

In general, packages that are being shipped by air via commercial and cargo carriers (such as Federal Express, DHL, and passenger aircraft) are affected by IATA regulations. These regulations are outlined in this section of the laboratory manual to provide examples of acceptable packaging procedures for infectious materials. However, because they may not reflect current national or IATA requirements for packaging and labeling for infectious substances, anyone packaging isolates or infectious specimens should consult the appropriate national regulations and the current edition of IATA *Dangerous Goods Regulations* before packing and shipping infectious substances by any means of transport. Tables 36a and 36b include images of labels and packages appropriate for shipping different classifications of packages under IATA regulations (current as of 2002). Note that a completed “Shipper’s Declaration for Dangerous Goods” form is required for shipments of hazardous materials including infectious substances; guidance in the use of this form is provided later in this appendix.

Definition of infectious substances

According to IATA [2003], infectious substances are defined as substances known or reasonably expected to contain pathogens. Pathogens are microorganisms (including bacteria, viruses, rickettsia, parasites, fungi) or recombinant microorganisms (hybrid or mutant) that are known or reasonably expected to cause infectious disease in humans or animals.

Definition of diagnostic specimens








According to IATA [2003], a diagnostic specimen is defined as any human or animal material being transported for diagnostic or investigational purposes. Human or animal material includes (but is not limited to) excreta, secretions, blood and its components, tissue and tissue fluids, and excludes live infected animals.

Diagnostic specimens are to be considered “diagnostic specimens” unless the source patient or animal has or may have a serious human or animal disease which can be readily transmitted from one individual to another, directly or indirectly, and for which effective treatment and preventative measures are not usually available, in which case they must be classified as “infectious substances.”

Guidelines for packaging and labeling infectious substances

Persons who ship infectious agents or diagnostic specimens must comply with all local and international regulations pertaining to the packaging and handling of these materials. They must ensure that specimens arrive at their destination in good condition and that they present no hazard to persons or animals during transport.

TABLE 36a: Summary of labels and markings required for safe and proper shipping of different types of packages

Package Type:								(Figure within table)
Diagnostic Specimens	3		3					Figure A
Diagnostic Specimens on Dry Ice	3		3					Figure B
Infectious Substance ...with less than 50 ml ...with 50 ml or more	3 3	3* 3*		3 3			3 3	Figure C Figure D
Infectious Substance on Dry Ice ...with less than 50 ml ...with 50 ml or more	3 3	3* 3*		3 3			3 3	Figure E Figure F
Dry Ice	3			3				Figure G

* If overpack used

TABLE 36b: Description of individual labels and markings required for safe and proper shipping of different types of packages


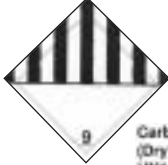

	<p>This orientation label should clearly mark which side is 'Up.' Two labels are required on all boxes, each one on opposite sides of the package.</p>
<div style="border: 1px solid black; padding: 5px; text-align: center;"> <p>Inner Packages Comply With Prescribed Specifications</p> </div>	<p>This marking must appear on an overpack when the regulations require the use of packagings bearing UN Specification Markings.</p>
<div style="border: 1px solid black; padding: 5px;"> <p>DIAGNOSTIC SPECIMENS</p> <p>Not Restricted, Packed in Compliance With IATA Packing Instruction 650</p> </div>	<p>This marking is required when shipping diagnostic specimens.</p>
<div style="display: flex; align-items: center;">  <div style="margin-left: 10px;"> <p>g</p> <p>Carbon dioxide, solid (Dry ice) UN1845 _____ kg.</p> </div> </div>	<p>These two labels are required when shipping a substance or specimen on dry ice.</p>
<div style="display: flex; align-items: center;">  <div style="margin-left: 10px;"> <p>INFECTIOUS SUBSTANCE</p> <p>Category _____</p> <p>UN _____</p> <p>_____ kg.</p> </div> </div>	<p>These three labels are required when shipping infectious substances. Please note when shipping infectious substances you <i>must</i> use UN certified 6.2 Infectious Substances Packaging.</p>

TABLE 36b: *continued*



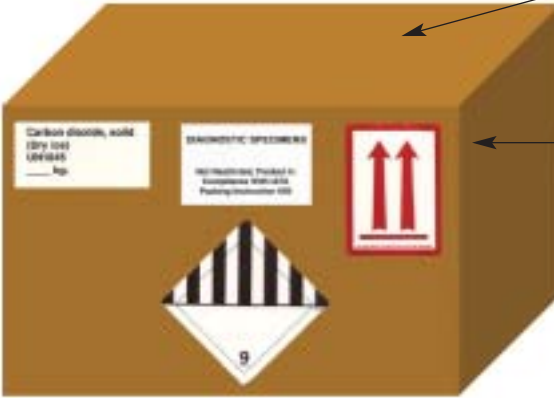
	<p>This label is required when shipping ≥ 50 ml of an infectious substance.</p>
<p><i>Figure A: Package with diagnostic specimens</i></p>	
	<p>Surface to which air waybill and/or address labels are affixed</p> <p>Must have two "up" arrows on opposite sides</p>
<p><i>Figure B: Package with diagnostic specimens on dry ice</i></p>	
	<p>Surface to which air waybill and/or address labels are affixed</p> <p>Must have two "up" arrows on opposite sides</p>

Figure C: Overpack with <50 ml of infectious substance

Label indicating name and telephone number of person responsible for shipment



Surface to which air waybill and/or address labels are affixed

Must have two "up" arrows on opposite sides

You must use UN certified 6.2 Infectious Substances Packaging.

Figure D: Overpack with ≥ 50 ml of infectious substance

Label indicating name and telephone number of person responsible for shipment



Surface to which air waybill and/or address labels are affixed

Must have two "up" arrows on opposite sides

You must use UN certified 6.2 Infectious Substances Packaging.

Figure E: Overpack with <50 ml infectious substance and dry ice

Label indicating name and telephone number of person responsible for shipment



Surface to which air waybill and/or address labels are affixed

Must have two "up" arrows on opposite sides

You must use UN certified 6.2 Infectious Substances Packaging.

Figure F: Overpack with ≥ 50 ml infectious substance and dry ice

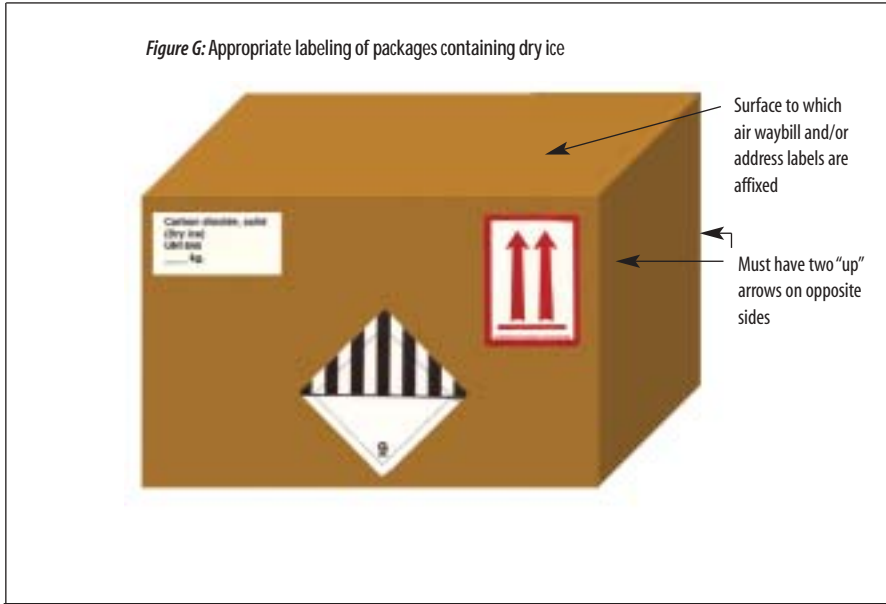
Label indicating name and telephone number of person responsible for shipment



Surface to which air waybill and/or address labels are affixed

Must have two "up" arrows on opposite sides

You must use UN certified 6.2 Infectious Substances Packaging.



The inner packaging of infectious substance shipments must include the following:

- An inner watertight primary container that is glass, metal, or plastic and has a leak-proof seal.
 - 3 Screw-cap tops should be reinforced with adhesive tape.
 - 3 Petri plates should not be shipped.
- A watertight, impact-resistant secondary container (*i.e.*, United Nations [UN] Specification Packaging that has been rigorously tested and certified for infectious substances)
- Absorbent material between the primary container and the secondary container.
 - 3 If multiple primary containers are placed in a single secondary packaging, they must be wrapped individually to ensure that contact between them is prevented. The absorbing material, such as cotton wool, must be sufficient to absorb the entire contents of all primary containers.
- An itemized list of contents, placed between the secondary packaging and the outer packaging.

Multiple primary receptacles placed in a single secondary packaging must be wrapped individually or, for infectious substances transported in liquid nitrogen, separated and supported to ensure that contact between them is prevented. The absorbing material must be sufficient to absorb the entire contents of all primary receptacles.

The outer packaging of infectious substance shipments must meet the following requirements:

- Be of sufficient strength to adequately protect and contain the contents.
- Be at least 100 mm (4 inches) in its smallest overall external dimension, and of sufficient size to accommodate all labels to be placed on a single surface without overlapping.
- Be durably and legibly marked on the outside with the address and telephone number of the shipper and the consignee (*i.e.*, the intended recipient). The infectious substance label must be affixed to the outside of the outer container, and must bear the inscription, “Infectious substance. In case of damage or leakage immediately notify public health authority.” The secondary packaging for infectious substances must be marked with UN Specification Markings denoting that the packaging has been tested and certified for shipping infectious substances.
- Be marked with the infectious substance marking (UN 2814): “Infectious substance, affecting humans (Genus species {or technical name}) x total number of milliliters or grams.” The species can be specified, or else indicated as “spp.” Note that this marking can be written by hand and does not require a special adhesive label. Genus and species may be written with or without italics or underlining. For example:
 - “Infectious substance, affecting humans (*N. meningitidis*) x 5.0 ml”
 - or
 - “Infectious substance, affecting humans (*Streptococcus* spp.) x 5.0 ml”
 - or
 - “Infectious substance, affecting humans (HIV) x 0.5 ml”
- Be labeled with a set of two up-arrows (⇧⇧) on at least two opposite sides of the outer box to indicate the proper package orientation for the closures to be in the upright position. In addition to the double arrows on the sides, the top of the box may also be labeled with the statement “This End Up” or “This Side Up.”
- Be labeled with a “Cargo Aircraft Only” label if the total volume of the infectious substance per outer shipping container is ≥ 50 ml.

- Be marked with the name and telephone number of the person responsible for the shipment.

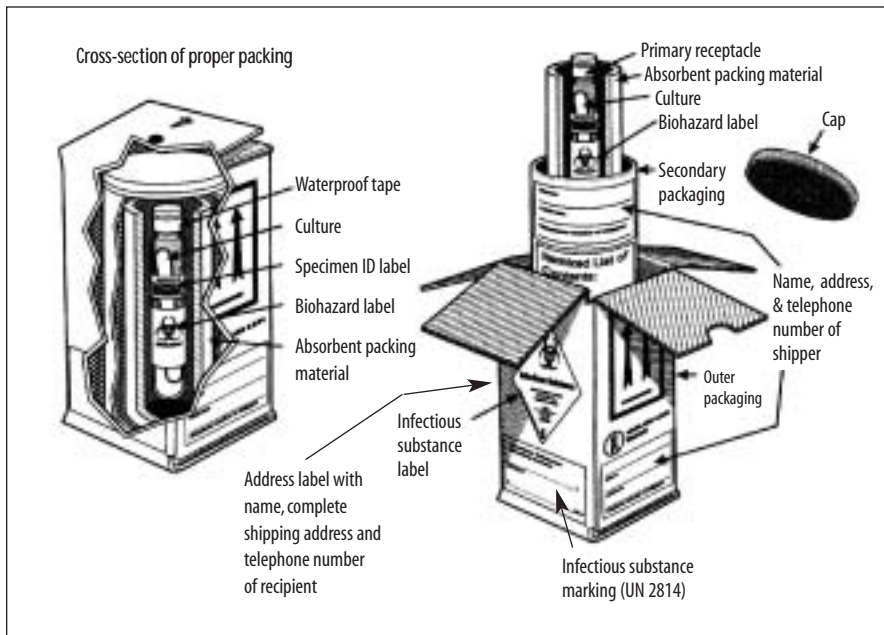
The packaging requirements for transport of infectious substances are illustrated in Figure 91.

Guidelines for packaging and labeling diagnostic specimens

Diagnostic (*i.e.*, clinical) specimens with a low probability of containing an infectious agent must be packaged as follows in packaging that will not leak after a 1.2-meter drop test procedure:

- Be “triple packed” with a watertight primary container, a leak-proof secondary container, and sufficient absorbent material in between the primary and secondary containers.
 - 3 The primary receptacle or the secondary packaging must be capable of withstanding, without leakage, an internal pressure differential of not less than 95 kiloPascals when between -40°C and +55°C. (Manufacturers indicate which of their packing and shipping containers meet these criteria.)
 - Infectious substance containers exceed these criteria and are therefore acceptable for use for packing and shipping of diagnostic specimens.
- Contain an itemized list of contents between the secondary packaging and the outer packaging.

FIGURE 91: Proper packing and labeling of the secondary container for shipping of infectious substances



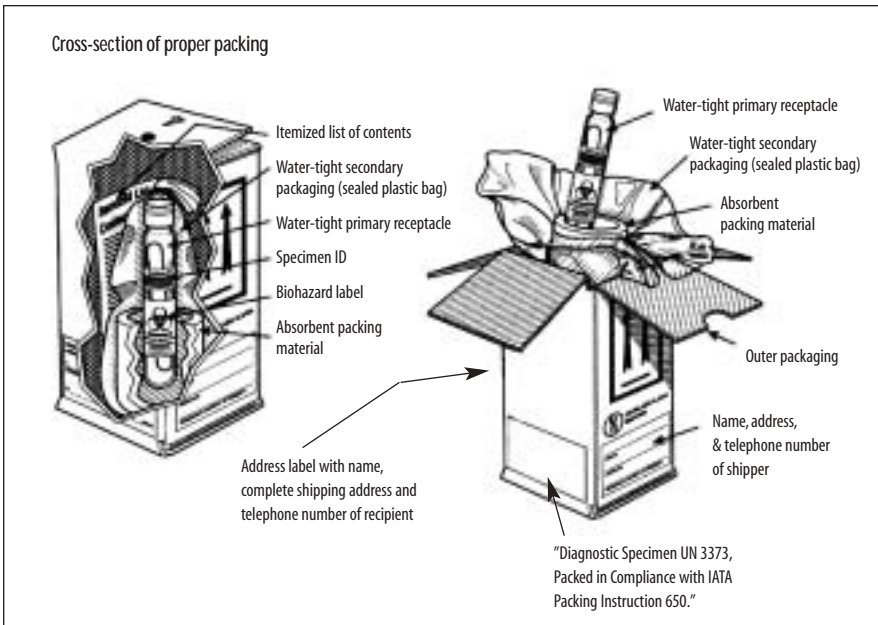
- Be marked with the diagnostic specimens statement on the outside of the outer container: “Diagnostic specimen. UN 3373. Packed in compliance with IATA Packing Instruction 650.” Note that this marking can be written by hand and does not require a special adhesive label.
- 3 If being shipped by air, the diagnostic specimens statement (“Diagnostic specimen. UN 3373. Packed in compliance with IATA Packing Instruction 650.”) must be present on the air waybill as well as on the outer container.

The packaging requirements for transport of diagnostic specimens are illustrated in Figure 92.

Guidelines for packaging and labeling of specimens shipped on dry ice (CO₂)

Wet ice or dry ice must be placed outside the secondary packaging in an overpack, and interior supports must be provided to secure the secondary packaging in the original position after the ice has dissipated. If wet ice is used, the packaging must be leak-proof. If dry ice is used, it must be packed according to IATA Packing Instruction 904: the outer packaging must permit the release of carbon dioxide [CO₂] gas. Cardboard and polystyrene (*i.e.*, Styrofoam) are two examples of materials suitable for the packaging of dry ice. In a temperate climate, approximately 6 pounds of dry ice will dissipate in a 24-hour period, and therefore at least that much (and preferably more) dry ice is suitable for a 24-hour shipment/delivery period; this amount should be adjusted accordingly for warmer

FIGURE 92: Proper packing and labeling of the secondary container for shipping of diagnostic specimens



climates and size of the box. The larger the box, the more dry ice required to keep the contents frozen. Note that for air transport, the maximum dry ice allowed in a single outer container is 200 kg (approximately 440 pounds).

Packages containing dry ice **must be properly marked** with the words “Carbon dioxide, solid (dry ice); UN1845; (and net weight of the dry ice in kg),” and a pre-printed Class 9 “Miscellaneous Dangerous Goods” label, as shown in Table 36.

When an overpack is used, the overpack must be marked with the statement “Inner packages comply with prescribed specifications” (because the UN Specification Markings will not be visible on the outer-most packaging).

Guidelines for completion of the “Shipper’s Declaration for Dangerous Goods” form

All shipments of hazardous materials including infectious substances must be accompanied by two original, completed copies of the “Shipper’s Declaration for Dangerous Goods” form, inserted in the pouch along with the other shipping documents. A sample Shipper’s Declaration for Dangerous Goods form with information required for completion is presented in Figure 93. It is important to remember the following in order to reduce the risk of a shipment being refused and returned to the laboratory of origin:

- International regulations require the diagonal hatch marks in the left and right margins to be **printed in red**, and so photocopies of this form may not be used.
- The form **must be completed in English**, although translations may accompany it on the same form.
- Specific terms, spellings, and nomenclature must be used. For example, a cardboard box **must** be referred to as “fibreboard box” (spelled with R before E), and there **must** be a comma after the term “infectious substance” within the statement “infectious substance, affecting humans” (Figure 93).
- The person responsible for the shipment must be listed in one of the address boxes; if the person responsible for the shipment is different than the shipper or recipient, include the responsible person’s telephone number alongside the name.
- Under the “Transport Details” portion of the form, cross out the option that does not apply.
 - If the shipment is **under 50 ml**, cross out “**cargo aircraft only.**”
 - If the shipment is **50 ml or more**, cross out “**passenger and cargo aircraft.**”
- Under the “Nature and Quantity of Dangerous Goods” portion of the form:
 - 3 The proper shipping name for infectious substances is “Infectious substance, affecting humans (technical name).” The technical name of the infectious

substance(s) **must** be included in parentheses after the proper shipping name; however, the specific species is not required, and “spp.” may follow the genus. It is therefore appropriate for the technical name of the infectious substance *Neisseria meningitidis* to be listed as either “(*Neisseria meningitidis*)” or “(*Neisseria* spp.)” Italics are permitted for the genus and species names but are not necessary.

- 3 For “Infectious substances, affecting humans (technical name)”: the proper class is 6.2; the UN number is UN2814; and the packing instruction is 602.
 - 3 For “Carbon dioxide, solid (dry ice)”: the proper class is 9; the UN number is UN1845; the packing group is III; and the packing instruction is 904.
 - 3 For infectious substances, the quantity must be noted in ml under the “Quantity and Type of Packing” portion of the form.
 - 3 For dry ice, the quantity must be noted in kg (measured in whole numbers) under the “Quantity and Type of Packing” portion of the form.
 - 3 If the UN specification marking is not visible on the outer package, the declaration must contain the words “OVERPACK USED” under the “Quantity and Type of Packing” portion of the form.
- Under the “Additional Handling Information” portion of the form, the 24-hour emergency contact telephone number must be answered by a person knowledgeable about emergency response procedures for damaged and leaking boxes.
 - The “Shipper’s Declaration for Dangerous Goods” form is a legal document and must be signed.

Be certain to contact the intended recipient prior to shipment of the box to share all shipping details, and make arrangements for proper handling during shipping and legal importation of the infectious substance without delay in delivery; these guidelines are in keeping with IATA regulation 1.3.3.1.

Reference publication for packing and shipping of dangerous goods

International Air Transport Association *Dangerous Goods Regulations* 44th Edition, Effective January 1, 2003. Montreal - Geneva.

FIGURE 93: Information required for proper completion of the "Shipper's Declaration for Dangerous Goods" form

SHIPPER'S DECLARATION FOR DANGEROUS GOODS (Printable at least two copies to the consignee)

<p>Shipper</p> <p>Name of Shipper Company Name Complete address (no P.O. Boxes) Telephone number (include area code) Person responsible (name and telephone)</p>	<p>Air Waybill No. ← use Air Waybill number of package</p> <p>Page of Pages ← REQUIRED</p> <p>Shipper's Reference Number (optional)</p>																								
<p>Consignee</p> <p>Name of Recipient Company Name Complete address (Not a P.O. Box) Telephone number (include area code) Person responsible (name and telephone)</p>	<p>Cross-out choice that does NOT apply (infectious substances are usually non-radioactive)</p> <p>red markings are REQUIRED</p>																								
<p>Two completed and signed copies of this Declaration must be handed to the operator</p>																									
<p>TRANSPORT DETAILS</p> <p>This shipment is written for the mode(s) specified for which it is applicable.</p> <p>Mode(s): <input type="checkbox"/> PASSENGER AND CARGO AIRCRAFT ONLY <input type="checkbox"/> CARGO AIRCRAFT ONLY</p> <p>Airport of Origin: _____ City, State, Country</p> <p>Airport of Destination: _____ City, State, Country</p>	<p>WARNING</p> <p>Failure to comply in all respects with the applicable Dangerous Goods Regulations may be in breach of the applicable law, subject to legal penalties. This Declaration must not, in any circumstances, be completed and/or signed by a consolidator, a forwarder or an IATA cargo agent.</p> <p>Shipment type (select one option): <input type="checkbox"/> (NON-RADIOACTIVE) <input type="checkbox"/> (RADIOACTIVE)</p>																								
<p>NATURE AND QUANTITY OF DANGEROUS GOODS</p>																									
<p>Dangerous Goods Identification</p>																									
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 35%;">Proper Shipping Name</th> <th style="width: 10%;">Class or Division</th> <th style="width: 10%;">ID or ID No.</th> <th style="width: 10%;">Packing Group</th> <th style="width: 10%;">Substance Risk</th> <th style="width: 15%;">Quantity and Type of packing</th> <th style="width: 10%;">Packing Inst.</th> <th style="width: 10%;">Authorization</th> </tr> </thead> <tbody> <tr> <td>Infectious substance, affecting humans (GENUS SPECIES)</td> <td>6.2</td> <td>UN2814</td> <td></td> <td></td> <td>1 FIBREBOARD BOX x _____ ml</td> <td>602</td> <td></td> </tr> <tr> <td>Carbon dioxide, solid (Dry ice)</td> <td>9</td> <td>UN1845</td> <td></td> <td></td> <td>1 FIBREBOARD BOX x _____ ml</td> <td>904</td> <td></td> </tr> </tbody> </table>	Proper Shipping Name	Class or Division	ID or ID No.	Packing Group	Substance Risk	Quantity and Type of packing	Packing Inst.	Authorization	Infectious substance, affecting humans (GENUS SPECIES)	6.2	UN2814			1 FIBREBOARD BOX x _____ ml	602		Carbon dioxide, solid (Dry ice)	9	UN1845			1 FIBREBOARD BOX x _____ ml	904		<p>If UN specification markings are not visible because the overpack covers the secondary packaging, include: OVERPACK USED</p>
Proper Shipping Name	Class or Division	ID or ID No.	Packing Group	Substance Risk	Quantity and Type of packing	Packing Inst.	Authorization																		
Infectious substance, affecting humans (GENUS SPECIES)	6.2	UN2814			1 FIBREBOARD BOX x _____ ml	602																			
Carbon dioxide, solid (Dry ice)	9	UN1845			1 FIBREBOARD BOX x _____ ml	904																			
<p>Additional Handling Information</p> <p>Prior arrangements as required by the ICAO and IATA Dangerous Goods Regulations 1.3.3.1 have been made ← REQUIRED</p>																									
<p>I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name and are classified, packaged, marked and labelled/cleared, and are in all respects in proper condition for transport according to applicable international and national governmental regulations.</p>																									
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Manufacturer, Supplier, and Distributor Contact Information

The following list of the manufacturers, suppliers, and distributors of the commonly used media and reagents does not indicate endorsement of these products and/or manufacturers. Note that contact information may change.

Follow the manufacturer's instructions closely when using commercially available media and reagents, and perform quality control activities regularly as appropriate.

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Dartford, Kent DA1 5LR, United Kingdom
Phone: (+44) 132 227 7711
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Norcross, GA 30071, USA
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Phone: (+33) 1 45 14 85 00

info@fr.vwr.com

Quality control strains

Many laboratories purchase QC strains from official culture collections, including the American Type Culture Collection (ATCC) and the National Collection of Type Cultures and Pathogenic Fungi (NCTC). This manual presents the ATCC numbers for quality control strains, but ATCC strains may also be obtained from the NCTC.

American Type Culture Collection (ATCC)

12301 Parklawn Drive, Rockville, MD 20852 USA

Phone (+1) 703-365-2700

Fax (+1) 703-365-2701

E-mail help@atcc.org

Internet <http://www.atcc.org>

National Collection of Type Cultures and Pathogenic Fungi (NCTC)

Public Health Laboratory Service, London NW9, England

E-mail nctc@phls.nhs.uk

Internet <http://www.phls.co.uk/services/nctc/>

Quality control strains also may be purchased from commercial companies such as Lab M. **Lab M** Topley House, 52 Wash Lane, Bury, BL9 6AU, England.

Etest® strips

Etest® strips may be somewhat more difficult to obtain than antimicrobial disks, and so specific information is included here regarding their acquisition. Etest® strips are available from:

AB BIODISK	AB BIODISK North America, Inc	Remel Inc. (Distributor)
Dalvagen 10	200 Centennial Ave	12076 Santa Fe Dr.
S 169 56	Piscataway, NJ. 08854-3910	Lenexa, KS 66215
Solna, Sweden	Phone: (+1) 732 457 0408	Phone: (+1) 913 888 0939
Phone: (+46) 8 730 0760	Fax: (+1) 732 457 8980	Fax: (+1) 913 888 5884
Fax: (+46) 8 83 81 58		

Find AB Biodisk on the Internet at: <http://www.abbioidisk.com>

In some cases discounts on Etest® strips may be available for projects funded by the World Health Organization (WHO), particularly for laboratories in resource-poor regions. To learn more about potential discounts, contact: Anne Bolmstrom, President AB BIODISK, at the company address in Sweden provided here.

International Reference Laboratories

Persons wishing to send isolates to an international reference laboratory for confirmation must **contact the laboratory prior to the packaging and shipping process in order to obtain information about import permits and to ascertain the laboratory is able to accept the shipment.** (*Note:* instructions for the proper packaging of isolates are found in Appendix 12.)

WHO Collaborating Centre for Research, Training, and Control in Diarrhoeal Diseases
International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)
G.P.O. Box 128
Dhaka 100
BANGLADESH

WHO Collaborating Centre for Diarrhoeal Diseases Research and Training
National Institute of Cholera and Enteric Diseases
P-33, CIT Road Scheme XM
Beliaghata
P.O. Box 177
Calcutta 700 016
INDIA

WHO Collaborating Centre for *Shigella*
National Reference Laboratory for *Escherichia coli* and *Shigella*
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C03
Atlanta, GA 30333 USA
Phone: (+1) 404 639 3344
Fax: (+1) 404 639 3333
E-mail: nas6@cdc.gov

National Reference Laboratory for *Vibrio cholerae* O1 and O139
Epidemic Investigations and Surveillance Laboratory
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C-03
Atlanta, GA 30333 USA
Phone: (+1) 404 639 3344
Fax: (+1) 404 639 3333
E-mail: cab4@cdc.gov

WHO Collaborating Centre for Reference and Research on *Salmonella*
Institut Pasteur
28 rue du Docteur Roux
F-75724 Paris Cedex 15
FRANCE
Phone: (+33) 1 45 68 83 46
Fax: (+33) 1 45 68 82 28

WHO Collaborating Centre for Phage-typing and Resistance of Enterobacteria
Division of Enteric Pathogens
Central Public Health Laboratory
Colindale Avenue
London NW9 5HT
United Kingdom
Phone: (+44) 181 200 4400
Fax: (+44) 181 200 7874

WHO Collaborating Centre for Global Monitoring of Antimicrobial Resistant Bacteria
Nosocomial Pathogens Laboratory Branch
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS G-08
Atlanta, GA 30333 USA
Fax: (+1) 404-639-2256
E-mail: zoa6@cdc.gov (e-mail contact is preferred.)

WHO Collaborating Centre for Reference and Research on Meningococci
Attention: Prof. Dominique A. Caugant, Head
Norwegian Institute of Public Health
Geitmyrsveien 75
P.O. Box 4404 Nydalen
N-0403 Oslo
NORWAY
Phone: (+47) 22 04 23 11
Fax: (+47) 22 04 25 18

Unité du méningocoque, Centre Collaborateur OMS
(Meningococcal Unit, WHO Collaborating Centre)
Institut de Médecine Tropicale du Service de Santé des Armées
Attention: Dr. Pierre Nicolas
Parc du Pharo, B.P. 46
F-13998 Marseille-Armées
France
Phone: (+33) 4 91 15 01 15
Fax: (+33) 4 91 59 44 77
E-mail: imtssa.meningo@free.fr

WHO Collaborating Centre for STD and HIV
(Gonococcal Antimicrobial Surveillance Programme – Western Pacific Region)
The Prince of Wales Hospital,
Randwick, Sydney
Australia 2031
Phone: (+ 61) 2 9382 9079
Fax: (+ 61) 2 9398 4275
E-mail: j.tapsall@unsw.edu.au or limniosa@sesahs.nsw.gov.au

Gonococcal Antimicrobial Surveillance Program for Latin America and the Caribbean
Centre for Research in Biopharmaceuticals
Room 4170, Guindon Hall
University of Ottawa
451 Smyth Road
Ottawa, Canada K1H 8M5
Phone: (+1) 613 562 5800, ext. 8379
Fax: (+1) 613 562 5699
E-mail: GASPLAC@uottawa.ca

*Quality control strains for supplemental antimicrobial susceptibility testing of
Neisseria gonorrhoeae can be obtained from:*

Neisseria Reference Laboratory
Gonorrhea Research Branch, Building 1 South / Room B260
Centers for Disease Control and Prevention
1600 Clifton Rd NE
Atlanta, GA 30333 USA

Attention:

Dr. David Trees (Phone: (+1) 404 639 2134; Fax: 404 639 2310;
E-mail: DTrees@cdc.gov)

or

Dr. Joan S. Knapp (Phone: (+1) 404 639 3470; Fax: 404 639 3976;
E-mail: JKnapp@cdc.gov)

Resources for quality assurance

Laboratorians may also be interested in seeking reference information regarding quality assessment (Q/A). The World Health Organization maintains a website regarding international external Q/A schemes:

http://www.who.int/pht/health_lab_technology/ieqass.html .

As of 2002, the WHO international Q/A assessment scheme organizer for microbiology is:

WHO Collaborating Centre for External Quality Assessment in
Clinical Microbiology

Attention: Dr J. Verhaegen

University Hospital St Raphael
Leuven, Belgium

An additional internet-based resource for information useful to laboratories in resource-limited settings is the “Public Health Care Laboratory” website:

<http://www.phclab.com>.

The organization states a mission, “. . . to serve as a global resource and information exchange forum in support of laboratory services in resource-poor countries and thereby contribute to sustainable quality improvement. . . .”
PHCLab.com can be contacted by e-mail at: mail@phclab.com .

Selected References

Reference manuals

“Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera.” Centers for Disease Control and Prevention. Atlanta, Georgia, USA. 1999.

Copies of the above enterics manual can be obtained from:
Foodborne and Diarrheal Diseases Laboratory Section,
Centers for Disease Control and Prevention
1600 Clifton Road, NE MailStop C-03
Atlanta, GA 30333 USA
Fax: 404-639-3333

“Laboratory Methods for the Diagnosis of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*.” World Health Organization. 1999.

- *Copies of the above meningitis manual can be obtained from the World Health Organization, Geneva.*

NCCLS. *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement*. NCCLS document M100-S12 (ISBN 1-56238-454-6). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2002. (General NCCLS website: <http://www.nccls.org>)

NCCLS. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standards – Seventh Edition*. NCCLS document M2-A7 (ISBN 1-56238-393-0). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA 2000. (General NCCLS website: <http://www.nccls.org>)

NCCLS. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard – Fifth Edition*. NCCLS document M7-A5 (ISBN 1-56238-394-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA 2000. (General NCCLS website: <http://www.nccls.org>)

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Clinical Microbiology Procedures Handbook. American Society for Microbiology - Washington, DC USA: 1992.

Basic Laboratory Procedures in Clinical Bacteriology. WHO, Geneva: 2001.

Manual of Basic Techniques for a Health Laboratory. WHO, Geneva: 2001.

Reference manuals (pending publication by WHO)

Generic Protocol to Measure the Burden of Pneumonia and Pneumococcal Disease in Children 0 to 23 Months of Age. WHO, Geneva: Pending.

WHO Surveillance Standards for Antimicrobial Resistance : WHO, Geneva: Pending.

Shipping and packing reference

International Air Transport Association *Dangerous Goods Regulations* 44th Edition, Effective January 1, 2003. Montreal - Geneva.

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