

# Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World



*Haemophilus influenzae,*  
*Neisseria meningitidis,*  
*Streptococcus pneumoniae,*  
*Neisseria gonorrhoeae,*  
*Salmonella* serotype Typhi,  
*Shigella,* and  
*Vibrio cholerae*



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*Haemophilus influenzae, Neisseria meningitidis,  
Streptococcus pneumoniae,  
Neisseria gonorrhoeae, Salmonella* serotype Typhi,  
*Shigella*, and *Vibrio cholerae*

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## List of Abbreviations Used in This Document

APW	Alkaline peptone water
ASM	American Society for Microbiology
ATCC	American Type Culture Collection
BS	Bismuth sulfite agar
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CSF	Cerebrospinal fluid
CTA	Cystine trypticase agar
DCA	Desoxycholate citrate agar
DE	Dorset egg medium
DGR	Dangerous Goods Regulations (publication)
GC	<i>Neisseria gonorrhoeae</i> (or, gonococcus)
GN	Gram-negative broth
HE	Hektoen enteric agar
HIA	Heart infusion agar
Hib	<i>Haemophilus influenzae</i> serotype b
HTM	<i>Haemophilus</i> test medium
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
ICG	International Collaboration on Gonococci
KIA	Kligler iron agar
LIA	Lysine iron agar
MAC	MacConkey agar
MIC	Minimal inhibitory concentration
ML	Martin-Lewis medium

MTM	Modified Thayer-Martin medium
NAD	Nicotinamide adenine dinucleotide (V factor)
NCCLS	Formerly known as the “National Committee on Clinical Laboratory Standards,” NCCLS is an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.
NP	Nasopharyngeal
PBS	Phosphate buffered saline
QC	Quality control
RCF	Relative centrifugal force (measured in xg)
SEL	Selenite broth
SIM	Sulfide-indole-motility medium
SPS	Sodium polyanetholesulfonate
SS	<i>Salmonella-Shigella</i> agar
STGG	Skim-milk tryptone glucose glycerol medium
STI	Sexually transmitted infection
TCBS	Thiosulfate citrate bile salts sucrose agar
T-I	Trans-isolate medium
TSA	Tryptone-based soy agar
TSB	Tryptone-based soy broth
TSI	Triple sugar iron agar
UN	United Nations
WHO	World Health Organization
XLD	Xylose lysine desoxycholate agar

# Introduction

Respiratory and enteric diseases comprise a substantial proportion of the burden of morbidity and mortality in the developing world; acute respiratory infection and diarrheal illness are the top two killers of children less than five years of age worldwide. Reproductive tract pathogens cause uncomplicated infections of the mucosal membranes; however, if left untreated, infections with these pathogens can also lead to pelvic inflammatory disease, ectopic pregnancies and infertility, and may facilitate the transmission of HIV. Public health interventions such as access to safe water, improved sanitation, hygiene, immunizations, education, health communication, and access to acute medical care with appropriate case management have contributed to on-going improvements in health, and in social and economic development. One outcome of the increased availability of antimicrobial agents for symptomatic treatment of illness in hospitals and community environments, however, has been the emergence of antimicrobial resistance in pathogens of public health concern.

Antimicrobial resistance is an issue of great significance for public health at the global level. However, it is of particular concern in the developing world because fewer affordable and appropriate treatment options are readily available. It has become increasingly important to monitor patterns of resistance as the antimicrobial susceptibility of bacterial pathogens which contribute significantly to the burden of respiratory, febrile, reproductive tract, and diarrheal illness has declined. Because antimicrobial susceptibility testing is resource-intensive, the World Health Organization (WHO) recommends that only one or two reference laboratories in a country perform these tests. Until now, however, there has not been a technically appropriate source of standardized information for laboratory detection of antimicrobial resistance that is practical for use in regions with limited resources.

This laboratory manual focuses on seven bacterial pathogens of public health importance in the developing world: *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella* serotype Typhi, *Shigella*, and *Vibrio cholerae*. Methods for the isolation and identification of each of these bacterial agents from clinical specimens are presented, and

standardized antimicrobial susceptibility testing techniques and criteria for interpretation are described. To benefit from the information presented in this manual, laboratorians must have received training in proper basic microbiological techniques and be comfortable with such tasks as sterilization of instruments and media preparation. Flow charts of procedures and color figures of bacterial colonies and typical reactions have been provided as supplements to the text for ease of comparative identification. Procedural accuracy and methodological standardization are critical to the performance of antimicrobial susceptibility testing, and adherence to protocols of quality control is also vital to ensure that test results are valid and meaningful.

In order for a laboratory to successfully undertake isolation, identification, and antimicrobial susceptibility testing responsibilities, it must participate in on-going investments in materials, supplies, media, reagents, and quality control, along with periodic training of personnel and quality assessment or proficiency testing. Any deviations from antimicrobial susceptibility testing methods as described in the following pages may invalidate the test results. Antimicrobial susceptibility test methods must be performed as described according to internationally recognized clinical guidelines such as those provided by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis) in order to provide meaningful results for clinical and epidemiological interpretation. Laboratory staff must be afforded the appropriate time and resources to carry out the procedures described in this manual if the results are to be meaningful and applicable to clinical and policy decisions.

As resistance to antimicrobial agents in the pathogens causing these diseases grows and changes, strategies of response also must evolve. Resistant pathogens can translate to fewer treatable infections and thus higher morbidity and mortality, a drain on resources, and an obstacle to social, economic, and health development overall. Timely communication between the laboratory and public health officials is essential to the shaping of locally treatment appropriate policies; the data collected in the laboratory are crucial components of the decision-making process for clinical and public health policies.

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# Requirements of a Reference Laboratory

## FOR THE PERFORMANCE OF STANDARDIZED ANTIMICROBIAL SUSCEPTIBILITY TESTING

A reference laboratory differs from a clinical laboratory in that microbiologists are able to dedicate their time to confirmation and investigation of isolates sent in from other laboratories or hospitals and (for the purposes of this manual) then perform standardized antimicrobial susceptibility testing. This manual is written and intended for use in a reference laboratory or national central laboratory setting, where material resources are consistently quality controlled and available in sufficient quantities for regular testing of isolates. Reference laboratories must participate in a quality assurance program at least once per year and should also administer quality assurance programs for laboratories in their jurisdiction; the World Health Organization (WHO) encourages central public health laboratories in countries with limited resources to establish national quality assessment schemes and to participate in at least three surveys per year. Time, supplies, and personnel can be costly; as a result, it is anticipated that not every country will be able to support a reference laboratory meeting these requirements. A country that can not establish a reference laboratory should consult a regional or sub-regional reference laboratory for further guidance and for advice on where to send isolates requiring further investigation.

In order to carry out the standardized procedures referred to in this laboratory manual (and many others), the laboratory must be able to make ongoing investments in equipment, supplies, and human resources (*i.e.*, trained laboratorians). The Ministry of Health (or similar appropriate agency) should therefore ensure that its central public health laboratory has the following items of great importance:

- Laboratory space
- Trained laboratory technologists
- Water (purified either by filter system or distillation apparatus)
- Stable source of electricity
- Equipment
  - Water bath
  - Incubator
  - Refrigerator
  - Freezer
  - Autoclave
  - Vortex mixer
  - Labels and/or permanent marking pens
  - Materials for record-keeping (*e.g.*, laboratory log-books, and a computer with printer and Internet / e-mail access)
  - Antimicrobial disks and / or antimicrobial gradient agar diffusion tests (Etests®) (*depending on the organisms to be tested*)
- Standard laboratory supplies (*e.g.*, plates, tubes, pipettes, flasks, inoculating loops, other glassware or plasticware, rulers, bunsen burners or alcohol burners, pH meter, bleach, alcohol), media and reagents

It is also of considerable importance that the reference laboratory have an open line of communication with public health authorities, including ministries of health, professionals in the medical field, and policymakers. If the laboratory is responding to an epidemic that extends across borders, an outside public health agency (*e.g.*, the WHO) may become involved; in such situations, it is significant that data from the laboratory will enable better decision-making for clinical treatment and public health policy in more than one country.

# Bacterial Agents of Pneumonia and Meningitis

*Haemophilus influenzae*

*Neisseria meningitidis*

*Streptococcus pneumoniae*

## *Haemophilus influenzae*

### CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

**H***aemophilus influenzae* is a common etiologic agent of diseases such as pneumonia, meningitis, otitis media, and conjunctivitis. Meningitis caused by *H. influenzae* occurs almost exclusively in children less than five years of age, and most invasive *H. influenzae* disease is caused by organisms with the type b polysaccharide capsule (*H. influenzae* type b, commonly abbreviated as Hib). There are conjugate vaccines to prevent *H. influenzae* infections caused by serotype b, though they are not widely available in some parts of the world. No vaccines for the other serotypes or for unencapsulated strains have been developed. Although meningitis is the most severe presentation of disease, *H. influenzae* pneumonia causes more morbidity than *H. influenzae* meningitis.

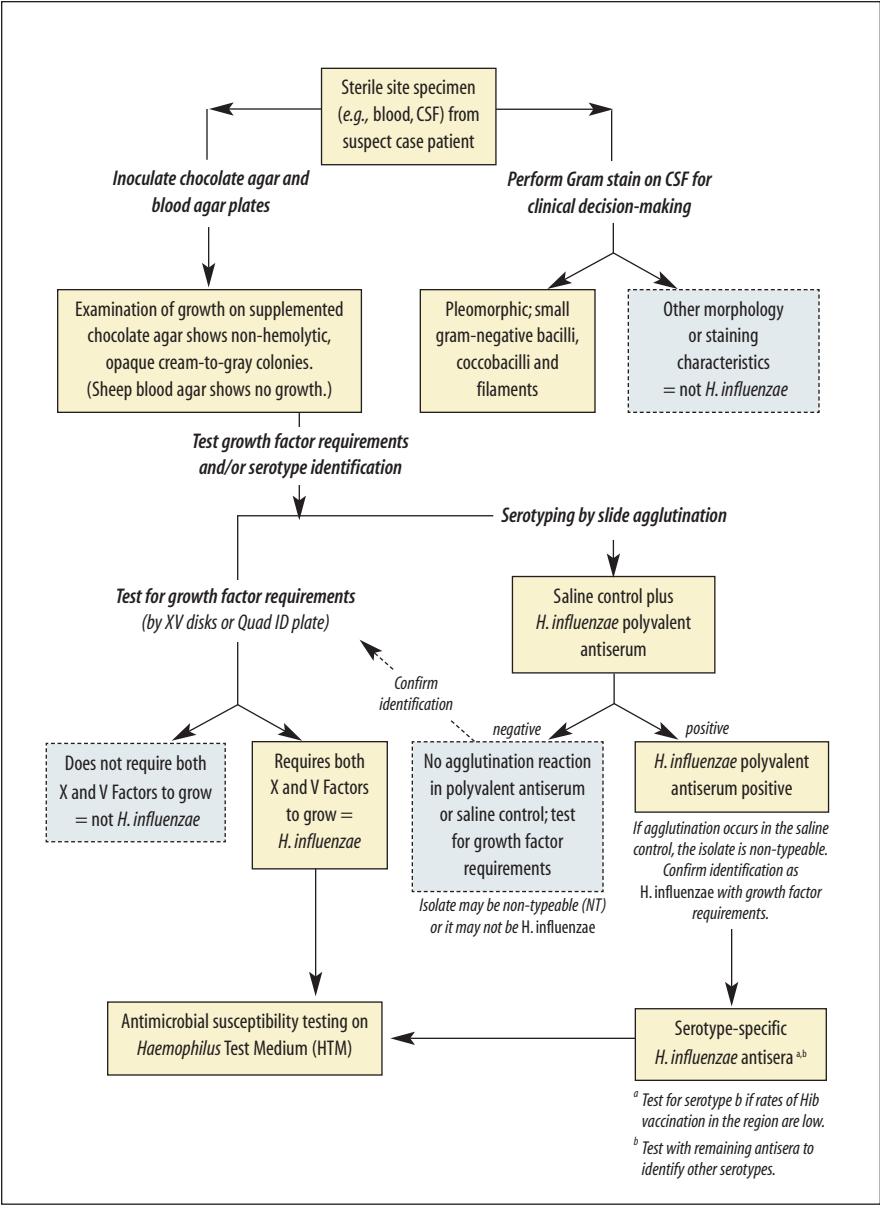
### Confirmatory identification of *H. influenzae*

*H. influenzae* are characterized as small, gram-negative bacilli or coccobacilli that require X and V growth factors, grow on chocolate agar (but **not** on sheep blood agar), and have a pungent indol smell. Methods for the isolation and presumptive identification of *H. influenzae* are included in Appendix 4. Figure 1 presents a schematic flowchart of confirmatory identification of *H. influenzae*.

#### Identification of the *H. influenzae* serotype

Laboratory identification of *H. influenzae* includes testing for X and V factor requirements and then performing serotyping; this sequence of testing is an efficient way to save costly antisera. However, when the laboratory results must be obtained rapidly for clinical decision-making, serotyping should be performed first the prompt presumptive identification of *H. influenzae*. Isolates identified as *H. influenzae* with typing antisera should still be confirmed by testing for X and V factor requirements.

FIGURE 1: Flowchart for laboratory identification of *Haemophilus influenzae*



*H. influenzae* is currently recognized to have six serotypes: a, b, c, d, e, and f. *H. influenzae* type b (Hib) is the major cause of both *H. influenzae* meningitis and of meningitis overall in unvaccinated children in many parts of the world. Suspected Hib isolates should be tested with Hib antiserum, an antiserum to one of the other groups, and saline. **A strongly positive (3+ or 4+) agglutination reaction with type b antiserum and no agglutination with antiserum to the other serotypes and saline is rapid evidence of Hib.**<sup>1</sup>

Antisera should be stored in the refrigerator at 4°C when not in immediate use. Screening an isolate first with polyvalent antiserum (which contains antisera to all six recognized serotypes) and a saline control is convenient and saves resources (*i.e.*, type-specific antisera).

- **If an isolate is positive in polyvalent antiserum** and negative in the saline control, proceed by testing the isolate with type b antiserum if Hib vaccination is uncommon in the patient's geographic region. If the serotype b reaction is negative, test with the remaining type-specific antisera (*i.e.*, a, c, d, e, and f).
  - If Hib disease is unlikely because of widespread vaccination, the culture should be tested with all the type-specific antisera (*i.e.*, a through f).
- **If an isolate is non-agglutinating in the polyvalent antiserum**, it is either non-typeable or is not *H. influenzae*. Therefore, growth factor requirements must be determined to confirm the identity of the isolate as *H. influenzae* or another species of *Haemophilus*.

#### ***Slide agglutination test for serotyping suspected H. influenzae isolates***

- a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slide into equal sections (*e.g.*, three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.
- b) Collect a small portion of growth from the surface of an overnight culture on chocolate agar (**without bacitracin**), a *Haemophilus* ID plate, or *Haemophilus* test medium (HTM) plate with a sterile inoculating loop. Make a moderately milky suspension of the test culture in a small vial with 250 µl (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible.
  - If only working with several isolates, another option is to make the suspension directly on the slide in 10 µl of formalinized physiological saline per droplet.

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<sup>1</sup> Laboratorians are often tempted to test suspect *H. influenzae* isolates only with type b antiserum since because serotype b (Hib) is vaccine preventable; however, **it is of great importance to screen the isolate with a saline control and at least one other antiserum in addition to type b.** Observing agglutination reactions with several antisera in different portions of the same slide permits comparisons and provides evidence that any agglutination in type b antiserum is not just a mild cross-reaction with a different serotype, providing the laboratorian and clinician with a more informed definition of a 'positive' reaction.

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a “moderately milky suspension” is roughly comparable to a 6 McFarland turbidity standard.
- c) For the agglutination reaction, use a micropipettor or a bacteriologic loop to transfer a drop (5–10 µl) of the cell suspension to the lower portion of two sections of the slide prepared in step a, above. Use enough suspension in the droplet so that it does not dry on the slide before testing with the antisera.
- d) Add 5–10 µl of polyvalent antiserum above the drop of suspension in one of the test sections on the slide.<sup>2</sup> In an adjacent section of the slide, use the same method to add a (5–10 µl) drop of saline above the final drop of suspension.
- **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.
- e) **Using a separate toothpick (or sterile loop) for each section**, mix the antiserum (and control saline) with the corresponding drop of cell suspension. Avoid contamination across the sections of the slide.
- f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, because it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the mixed drops and read the slide agglutination reactions under bright light and over a black background, as shown in Figure 2.
- g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figures 11 and 42). When a strain reacts with more than one antiserum, or agglutinates in saline, the result is recorded as non-typeable.
- *If strong agglutination occurs in the polyvalent antiserum:* Using the methods described in steps a through f (above), continue testing the isolate with type b antiserum and other type-specific antisera to identify the serotype.
  - *If agglutination does not occur in the polyvalent antiserum:* The isolate is either non-typeable or not *H. influenzae*. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

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<sup>2</sup> This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only approximately 5–10 µl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 µl of antisera are required for agglutination reactions to occur using the methods presented here, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

- *If agglutination occurs in the saline control:* The isolate is recorded as non-typeable. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

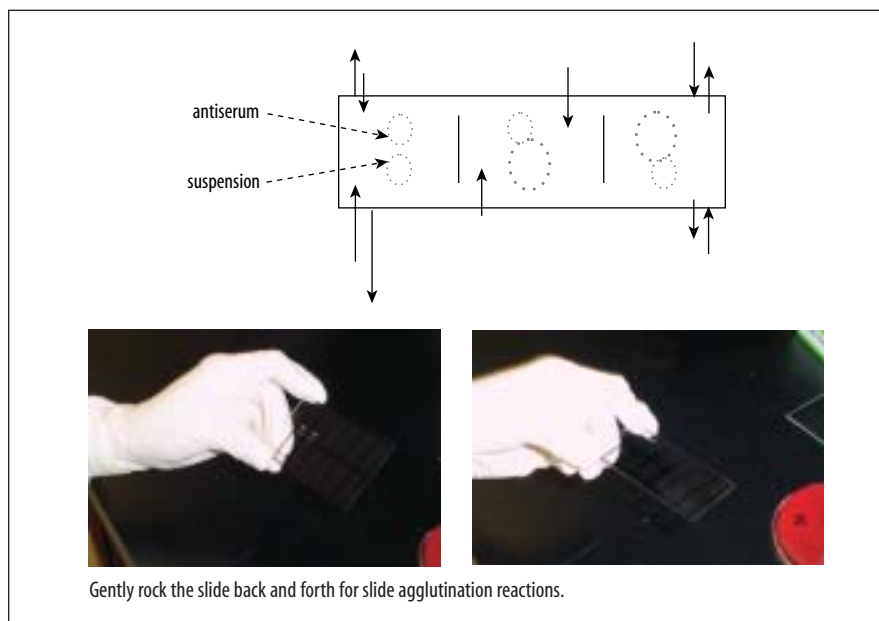
Record results and report to attending clinicians, as appropriate.

## Growth factor (X and V) requirements

*H. influenzae* is a fastidious organism requiring media containing haemin (X factor) and nicotinamide adenine dinucleotide (NAD, V factor) for growth. The standard medium is chocolate agar, which is often prepared with horse blood, a good source of both X and V factors (Appendix 2). Heating the blood is necessary to make both factors available to the organism. Chocolate agar with added supplements (e.g., IsoVitalEX, Supplement B, or Vitox) is available commercially or can be prepared in the laboratory. Supplemented chocolate agar is superior to unsupplemented medium for growth of *H. influenzae* and is the medium of choice. Although some strains of *H. influenzae* may grow on unsupplemented chocolate agar, **supplements must be added to reliably support the growth of most strains.**

*H. influenzae* is identified on the basis of its growth requirements for X and V factors (Table 1). *H. influenzae* can be differentiated from most other species of *Haemophilus* by its requirement for both X and V factors for growth.

FIGURE 2: Techniques to properly mix antiserum and suspension for slide agglutination



*H. haemolyticus* is the only other species requiring X and V factors but this species differs from *H. influenzae* by producing hemolysis on horse- or rabbit blood agar.

**Tests to identify X and V growth factor requirements: paper disks and strips or Quad ID plates**

Growth factor requirements can be identified with paper disks or strips (using the principles of agar diffusion) or by using Quad ID plates (which contain four types of media with and without X and V factors).

- **Growth factor test using X, V, and XV factor paper disks or strips**  
A medium completely without X and V factors, such as tryptone-based soy agar (TSA) or heart infusion agar (HIA), must be used for this test.

*Methods*

- a) Prepare a heavy suspension of cells (1 McFarland turbidity standard, see Appendix 2) from a primary isolation plate in a suitable broth (e.g., tryptone-based soy broth (TSB) or heart infusion broth). If the primary isolation plate contains insufficient growth or is contaminated, make a subculture on a chocolate agar plate. When preparing the broth **avoid transfer of agar medium to the broth**; even the smallest sample of agar will affect the test and **may lead to misidentification** of the bacteria because the agar contains X and V factors.
- b) Inoculate a HIA or TSA plate. A sterile swab or sterile loop of the suspension should be streaked over one-half of the plate (with streaking in at least two directions to ensure confluent growth). Two strains can be tested on one 100-mm plate, but care must be taken to ensure the isolates do not overlap. Paper strips or disks containing X, V, and XV factors are placed on the inoculated plate after the inoculum has dried. **When two bacterial strains are tested on the same plate, as shown in Figure 3, the disks should be placed in the exact manner shown.**
- c) Carefully invert the plate and place it in a CO<sub>2</sub>-incubator or candle-extinction jar. Incubate it for 18–24 hours at 35°C. *H. influenzae* will grow

TABLE 1: Identification of *Haemophilus* species by their growth requirements

Species	X- and V-Factor Requirements		β-hemolysis on rabbit blood agar
	X	V	
<i>H. influenzae</i>	+	+	–
<i>H. parainfluenzae</i> *	–	+	–
<i>H. haemolyticus</i>	+	+	+
<i>H. parahaemolyticus</i>	–	+	+
<i>H. aphrophilus</i>	+	–	–
<i>H. paraphrophilus</i> *	–	+	–

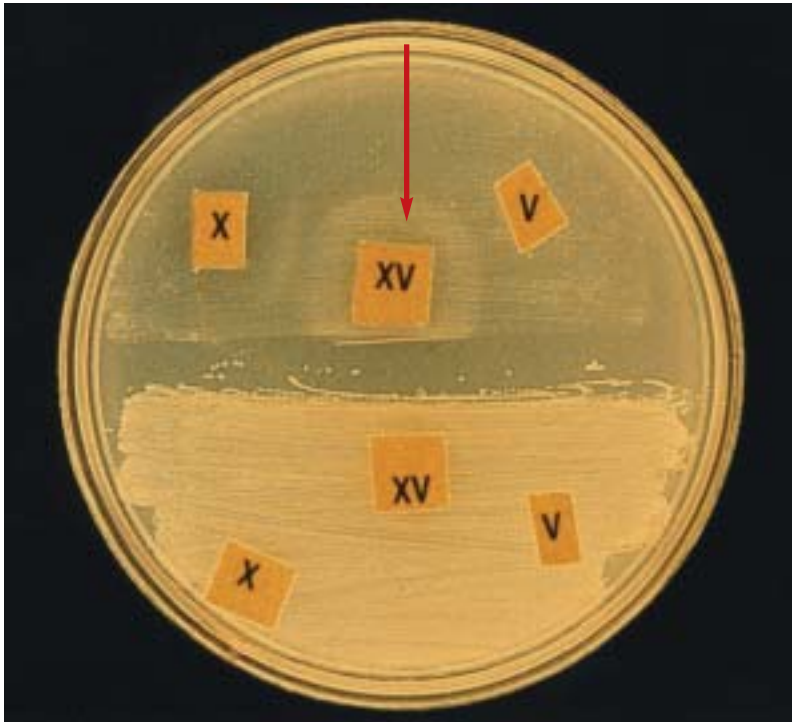
\* *H. paraphrophilus* is ornithine negative, whereas *H. parainfluenzae* is ornithine positive.

only around the XV disk (*i.e.*, the disk containing both X and V factors), as shown on the upper half of the plate in Figure 3.

- **Growth factor test using *Haemophilus* Quad ID Plates**

Quad ID plates are another, although more expensive, method for determining growth factor requirements of *Haemophilus* isolates (Figure 4). Available commercially, the Quad ID plate is divided into four agar quadrants: one quadrant includes medium containing haemin (X factor); one quadrant includes medium containing NAD (V factor); another quadrant contains medium that includes both X and V factors; and, the final quadrant contains heart infusion agar or blood agar base with 5% horse blood for differentiating *H. haemolyticus*, an oral species requiring X and V factors, from *H. influenzae*. Quadrant location of the growth factors may vary with commercial brand of the Quad ID plate.

FIGURE 3: Growth factor requirements: X and V factors on paper disks



The top strain is only growing around the disk containing both X and V factors and can therefore be considered presumptive *H. influenzae*.

FIGURE 4: Growth factor requirements: *Haemophilus* Quad ID plate



### Methods

- a) Inoculate the Quad ID plate by suspending the growth from a young, pure culture of suspected *Haemophilus* in tryptone soy broth (TSB) or distilled water to a light, milky suspension (equivalent to a 0.5 McFarland turbidity standard). Using a bacteriological loop, streak one loopful of this suspension on each quadrant of the plate, beginning with the V quadrant and ending with the blood quadrant. Streak the entire quadrant, starting at the periphery and streaking toward the center of the plate. Stab into the blood agar for detection of weak hemolysis.

- b) Invert the plate and incubate under a CO<sub>2</sub>-enhanced atmosphere (in a candle-jar or CO<sub>2</sub>-incubator) for 18–24 hours at 35°C.
- c) After incubation, examine the blood section for hemolysis and the other sections for growth. *H. influenzae* typically shows growth in the XV quadrant and in the (horse-) blood quadrant with no hemolysis. If strong growth occurs in either one of the X or V quadrants besides XV, the organism is probably another species of *Haemophilus*. If growth occurs in every quadrant, the culture is probably not a species of *Haemophilus*. (**Note:** Occasionally, *H. influenzae* may show slight growth in the V-factor quadrant.) Read and record results.

### Hemolytic reactions of *Haemophilus* species

Although most laboratories will not need to determine the hemolytic reaction of each *Haemophilus* spp. (because too few *Haemophilus* strains will be isolated), some laboratories may want to determine the hemolytic reaction to definitively identify both *H. influenzae* and *H. haemolyticus*.

- If X, V, and XV factor disks or strips were used to test growth factor requirements, a separate test to detect hemolytic reactions must be performed by inoculating a broth suspension of the strain on HIA + 5% rabbit blood (or agar infusion base containing horse blood); the hemolytic reaction permits determination the species.
- If a Quad ID plate was used to test for growth factor requirements, the hemolytic reaction of the organism is tested in the (horse-) blood agar quadrant of the plate; thus no separate test is required.

*H. influenzae* should be  $\alpha$ -hemolytic (*i.e.*, causing a greening in the agar around the colony) or  $\gamma$ -hemolytic (non-hemolytic) on the HIA plate containing 5% rabbit blood, while *H. haemolyticus* will exhibit  $\beta$ -hemolysis (*i.e.*, a clearing of the blood cells in the agar surrounding the colonies on the plate). A summary of test results used in the identification of *H. influenzae* and most closely related *Haemophilus* species is shown in Table 1. Proper determination of the hemolytic reaction is the only way to differentiate *H. influenzae* from *H. haemolyticus*.

### Antimicrobial susceptibility testing of *H. influenzae*

The results of antimicrobial susceptibility tests will be used to select the most effective antimicrobial agent to use for treating patients. This laboratory manual describes susceptibility testing of *Haemophilus influenzae* by the disk diffusion method and by the antibiotic gradient strip (Etest®) testing method. Although disk diffusion will provide information as to whether a strain is susceptible,

intermediate, or resistant, the Etest® provides more detailed information about the minimal inhibitory concentration (MIC) of an antimicrobial agent. **The accuracy and reproducibility of these tests are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis.** A sample worksheet for recording antimicrobial susceptibility test results for *H. influenzae* is included in Figure 5.

## Media and disks for antimicrobial susceptibility testing

Antimicrobial susceptibility can be determined using the disk diffusion method. The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;<sup>3</sup> if performed precisely according to the following protocol, this method will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. The accuracy and reproducibility of this test are dependent on the consistent use of a standard set of procedures in laboratories. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results.

The recommended medium for antimicrobial susceptibility testing for *H. influenzae* is *Haemophilus* test medium (HTM) (Appendix 2). The Mueller-Hinton agar used for this test should be thymidine-free to obtain consistent results with trimethoprim-sulfamethoxazole (also referred to as cotrimoxazole). All media used for antimicrobial susceptibility testing should be freshly prepared. Recommended antimicrobial agents for testing are ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole.

The 10-µg ampicillin disk predicts both intrinsic (*i.e.*, penicillin-binding protein-mediated, or “PBP”) and  $\beta$ -lactamase (beta-lactamase) mediated penicillin and ampicillin resistance and should be used when testing *H. influenzae*. (Methods for  $\beta$ -lactamase testing of *H. influenzae* are listed after the direct antimicrobial susceptibility testing methods in this section.) For *H. influenzae*, a 30-µg chloramphenicol disk is used for predicting resistance to chloramphenicol, and a 1.25/23.75-µg trimethoprim-sulfamethoxazole disk is used for predicting trimethoprim-sulfamethoxazole resistance. **The zone diameter sizes can only be properly interpreted when HTM is used**, as per NCCLS standards.

## Quality control of antimicrobial susceptibility testing of *H. influenzae*

Quality control tests must be performed as part of the normal laboratory routine. To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. *H. influenzae* ATCC 49247 is

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<sup>3</sup> Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

FIGURE 5: Sample form for recording antimicrobial susceptibility test results for *Haemophilus influenzae*

Date of Testing: \_\_\_\_/\_\_\_\_/\_\_\_\_  
Test performed by: \_\_\_\_\_

Interpretation of susceptibility: S = susceptible I = intermediate R = resistant

Specimen number	Meningitis isolate?	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Ampicillin	(other drug)
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
ATCC 49247	N/A	<i>H. influenzae</i> NCCLS QC strain QC in range? <input type="checkbox"/> <input type="checkbox"/>	mm µg/ml S I R Yes No	mm µg/ml S I R Yes No	mm µg/ml S I R Yes No	mm µg/ml S I R Yes No

Revealed by: \_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

Note: After 16 – 18 hours of incubation, check the results for the quality control (QC) strain against the standard acceptable ranges; if they are within control limits, continue reading results for the test isolate. Record disk diffusion results in mm and MIC results in µg/ml. (Inhibition zone ranges and breakpoints for interpretation of results may be found in table 2.)

the control strain used when testing *H. influenzae* for most antimicrobial agents (e.g., ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole), although ATCC 49766 is appropriate for some others. (Consult NCCLS document M100-S12 [2002] for more complete information.) **Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 2.** If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

- **Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other environmental factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not.
- **If the depth of the agar in the plate is not uniformly 3–4 mm,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the pH of the test medium is not between 7.2 and 7.4,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, even if the isolates are susceptible, when colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks.

Quality control tests should be performed once per week if antimicrobial susceptibility tests are performed daily (after 30 days of in-control results), or with every group of tests when testing is done less frequently. They should also be done with each new batch of test medium and every time a new lot of disks is used.

### Antimicrobial susceptibility testing of *H. influenzae* by the disk diffusion method

Prepare the inoculum for seeding the antimicrobial susceptibility media with *H. influenzae* from fresh, pure cultures of *H. influenzae* (i.e., from isolates grown overnight on supplemented chocolate agar). Prepare cell suspensions of the bacteria to be tested in broth or sterile physiological saline; use a suspension equal to a density of a 0.5 McFarland turbidity standard for the inoculum. (Preparation of a McFarland turbidity standard is described in Appendix 2.)

- a) Suspend viable colonies from an overnight chocolate agar plate in a tube of broth to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells with the broth. **This suspension should be used within 15 minutes.**
  - b) Compare the suspension to the 0.5 McFarland turbidity standard by holding the suspension and the McFarland turbidity standard in front of a light against a white background with contrasting black lines and compare the density (see Figures 51 and 52). If the density of the suspension is too heavy, the suspension should be diluted with additional broth. If the density of the suspension is too light, additional bacteria should be added to the suspension.
  - c) When the proper density is achieved, dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid.
  - d) Use the swab to inoculate the entire surface of the HTM plate three times, rotating the plate 60 degrees between each inoculation (see Figure 34). Use the same swab with each rotated streak, but **do not re-dip the swab in the inoculum** (*i.e.*, the bacterial cell suspension).
  - e) Allow the inoculum to dry before the disks are placed on the HTM plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. (If drying takes longer than 15 minutes, use a smaller volume of inoculum in the future.)
  - f) After the plate is dry, antimicrobial disks should be placed on the HTM plate as shown in Figure 6. The disks should be placed on the agar with sterile forceps and tapped gently to insure adherence to the agar. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**
  - g) Invert the plate and incubate it in a CO<sub>2</sub>-enriched atmosphere (5% CO<sub>2</sub>-incubator or candle-extinction jar) for 16–18 hours at 35°C.
- **Note:** If this is a new batch of HTM, the antimicrobial disks are new, or it is an otherwise appropriate time to perform quality control, follow steps *a* through *g*- above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference quality control strain (for the antimicrobial agents included in this chapter) are presented in Table 2.
- h) After overnight incubation, measure the diameter of each zone of inhibition. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (Figure 6).
    - Care should be taken not to touch the disk or surface of the agar. Sterilize the ruler occasionally to prevent transmission of the bacteria. In all

measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony. Record the results in millimeters (mm). Figure 5 provides a sample form for recording results.

- i) Interpretation of the antimicrobial susceptibility is obtained by comparing the results obtained and recorded (in the manner described in this protocol) to the NCCLS standard inhibition zone diameter sizes presented in Table 2.

### Minimal inhibitory concentration testing of *H. influenzae* isolates

Laboratorians determining the minimal inhibitory concentration (MIC) for resistant isolates must be highly skilled in performing these tests and committed to obtaining accurate and reproducible results. In addition, a national (or regional) reference laboratory must have the ability and resources to store isolates either by lyophilization or by freezing at  $-70^{\circ}\text{C}$ .

Antimicrobial susceptibility testing by disk diffusion indicates whether an organism is susceptible or resistant to an antimicrobial agent. For surveillance purposes, a laboratory may want to quantify “intermediate” antimicrobial

FIGURE 6: The antimicrobial susceptibility disk diffusion test: disk placement and measurement of inhibition zone diameters

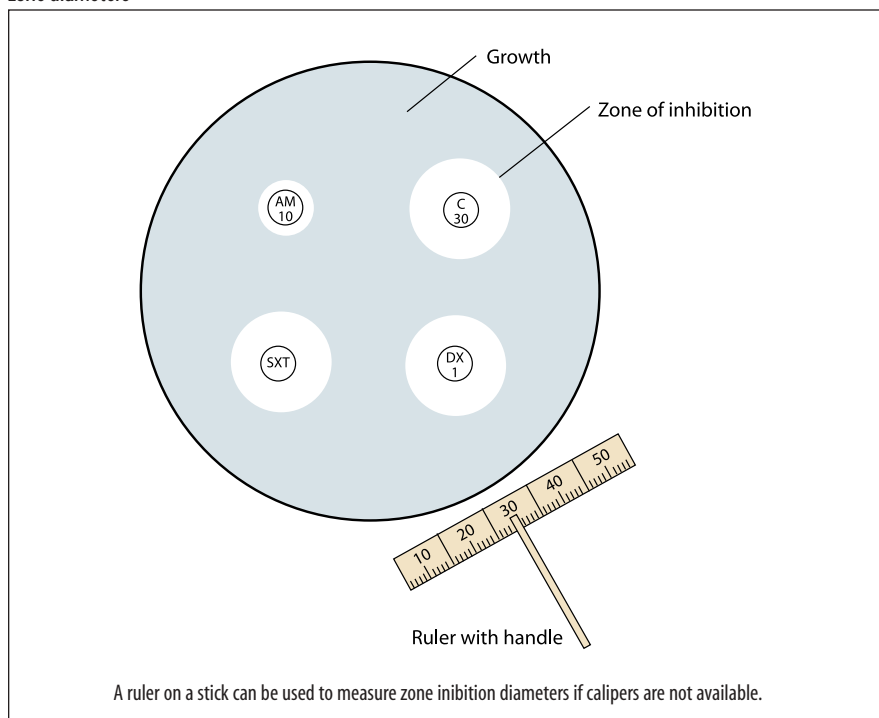


TABLE 2: Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for *Haemophilus influenzae*

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml) <sup>a</sup>			NCCLS QC strain <i>H. influenzae</i> ATCC 49247 <sup>b</sup>
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	> 29 mm (< 2 µg/ml)	26– 28 mm (4 µg/ml)	< 25 mm (> 8 µg/ml)	31 – 40 mm (0.25 – 1 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25/ 23.75 µg	≥ 16 mm (< 0.5/9.5 µg/ml)	11 mm – 15 mm (1/18 – 2/36 µg/ml)	≤ 10 mm (> 4/76 µg/ml)	24 – 32 mm (0.03/0.59 – 0.25/4.75 µg/ml)
Ampicillin	10 µg	≥ 22 mm (< 1 µg/ml)	19 mm – 21 mm (2 µg/ml)	≤ 18 mm (> 4 µg/ml)	13 – 21 mm (2 – 8 µg/ml)

<sup>a</sup> Source: NCCLS (2002) *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, PA 19087-1898 USA.

<sup>b</sup> The quality control strain *H. influenzae* ATCC 49247 is appropriate for the testing of the antimicrobial agents included in this table and this laboratory manual overall; however, for testing of some other antimicrobial agents, NCCLS recommends that a different QC strain be used. Laboratories testing the susceptibility of *H. influenzae* to antimicrobial agents other than those listed should therefore refer to the NCCLS document M100-S12 (or subsequent updates) for appropriate methods.

susceptibility test results to trimethoprim-sulfamethoxazole detected by disk diffusion testing with MIC testing.

MIC testing by dilution can be expensive and challenging; because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. In countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted as described earlier in this chapter.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest<sup>®</sup> serves as a test method that is both convenient and reliable.<sup>4</sup> The Etest<sup>®</sup> requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest<sup>®</sup> strips must be consistently stored in a freezer at -20°C.**

The Etest<sup>®</sup> is an antimicrobial susceptibility testing method that is as technically simple to perform as disk diffusion and produces semi-quantitative results that are measured in micrograms per milliliter (µg/ml). It is drug-specific, consists of a thin plastic antibiotic gradient strip that is applied to an inoculated agar plate, and is convenient in that it applies the principles of agar diffusion to perform semi-quantitative testing.<sup>5</sup>

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 log<sub>2</sub> dilutions by a conventional reference MIC procedure as suggested by the

<sup>4</sup> The Etest<sup>®</sup> can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

NCCLS. The Etest® has been compared with and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations (see, e.g. Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer's directions for use of the Etest®**, as certain antibiotic-bacteria (“drug-bug”) combinations have special testing requirements.

### ***Methods for antimicrobial susceptibility testing with the Etest®***

For *H. influenzae*, HTM is used when performing antimicrobial susceptibility testing. Follow the directions on the package insert included with the Etest® strips. Either 150-mm or 100-mm plates can be used, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate (see Figure 7).

- a) Suspend viable colonies from an overnight chocolate agar plate into a broth tube to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**
- b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- c) Allow the plate to dry for up to 15 minutes. **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20°C freezer and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20°C freezer.

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<sup>5</sup> Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

- d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps, oriented as shown in Figure 7. (Make sure that the printed MIC values are facing upward, [*i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar].) **Once applied, do not move the antimicrobial gradient strips.**
- e) Incubate the plates in an inverted position in a CO<sub>2</sub>-enriched atmosphere (2% – 5% CO<sub>2</sub>) for 16–18 hours at 35°C. A candle-extinction jar may be used if a CO<sub>2</sub>-incubator is not available.
- f) After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read the MIC at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,<sup>6</sup> and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a *H. influenzae* isolate to ampicillin, an MIC recorded from the graduations on the Etest® strip might be 0.75 mg/ml; however, the reported MIC would be 1.0 µg/ml.

Breakpoints for interpretation of MICs follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert**. NCCLS breakpoints for antimicrobial agents used for *H. influenzae* are included in Table 2.

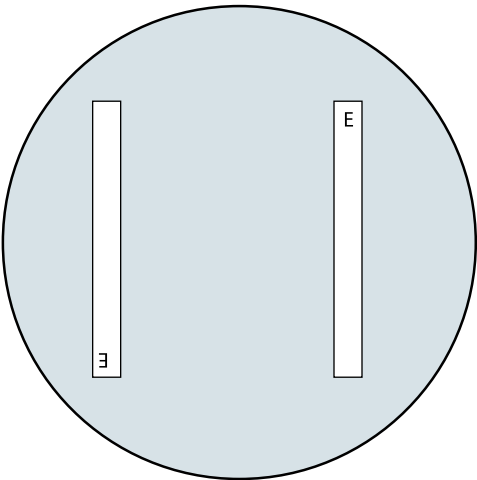
### Surveillance for emerging antimicrobial resistance in *H. influenzae*

Laboratories may wish to help detect the emergence of new strains of *Haemophilus* by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. A laboratory might look at specific drugs or characteristic groupings (such as, for example, β-lactamase negative, ampicillin resistant

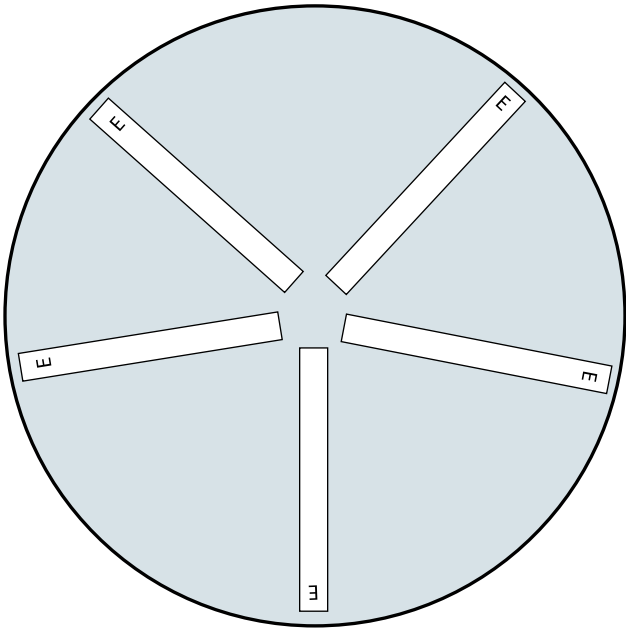
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<sup>6</sup> AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abbiobdisk.com>.

FIGURE 7: Proper placement of Etest® strips on dry, inoculated plates



Up to two Etest® strips can be placed on a 100 mm plate, as shown.

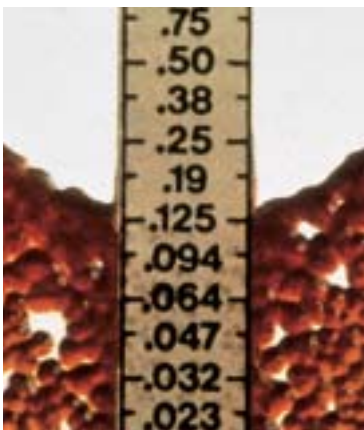


Up to five Etest® strips can be placed on a 150 mm plate, as shown.

FIGURE 8a: Guidance for reading Etest® results



If the strip is backwards,  
MIC = INVALID!  
Retest and position the strip with the MIC scale  
facing the opening of the plate.



Intersection in between markings.  
Read the next higher value. MIC 0.19 µg/ml.



Different intersections on either side of the strip.  
Read the higher value; if the difference is >1  
dilution, repeat the test. MIC 0.5 µg/ml.



Ignore a thin line of growth at the edge of the strip  
caused by organisms growing in a tunnel of water.  
MIC 0.25 µg/ml.

**The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).**

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FIGURE 8b: Guidance for reading Etest® results



Bacteriostatic drugs such as trimethoprim and sulphonamides can give diffuse edges. Read at 80% inhibition. MIC 3 µg/ml.



Isolated resistant colonies due to low-level mutation. MIC >256 µg/ml.



Paradoxical effect showing partial regrowth after an initial inhibition. MIC 8 µg/ml.



Induction of  $\beta$ -lactamase production by clavulanic acid at the higher MIC range. MIC 96 µg/ml.

**The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).**

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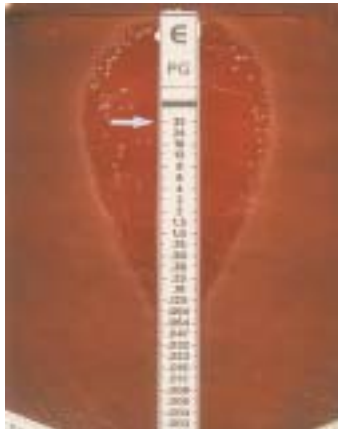
FIGURE 8c: Guidance for reading Etest® results



Tilt the plate to visualize pin-point colonies and hazes. This is particularly important for pneumococci. MIC 1 µg/ml.



Scrutinize pneumococcal end-points carefully to pick up all microcolonies. Tilt the plate and/or use a magnifying glass. MIC 2 µg/ml.



A highly resistant subpopulation in pneumococci. MIC >32 µg/ml.



Encapsulated strains may not give a confluent intersection. MIC 1 µg/ml.

**The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).**

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[BLNAR] *H. influenzae*). These strains are believed to be rare at present, but are of great interest to public health policy and clinicians because although they may exhibit *in vitro* susceptibility to certain drugs (e.g., amoxicillin + clavulanic acid, cefprozil, cefuroxime, and others), they should still be considered resistant *in vivo* [NCCLS 2002].

Testing for emerging resistance should not be done with each batch of antimicrobial susceptibility tests, nor with each new batch of media. Instead such testing could be done periodically (e.g., on an annual basis), for example on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11. Antimicrobials of interest could include (but are not necessarily limited to) ceftriaxone and fluoroquinolones. Appropriate zone diameter sizes can be found in NCCLS documents, which are updated regularly. **If any of these rare strains with reduced susceptibility are found in the course of this surveillance, notify an international reference laboratory and submit the isolate for further investigation.** A list of international reference laboratories is included in Appendix 14.

### **Testing *H. influenzae* for $\beta$ -lactamase production**

Testing the *H. influenzae* isolates for the presence of  $\beta$ -lactamase will identify most of ampicillin-resistant strains, because most (but not all) ampicillin resistance among *H. influenzae* is caused by the presence of  $\beta$ -lactamase. Several techniques are available for the detection of  $\beta$ -lactamases. All the tests are based on determination of breakdown products and use either a natural substrate (e.g., penicillin) or a chromogenic substance (e.g., nitrocefin). Two methods for detection of  $\beta$ -lactamase are presented in this manual: the nitrocefin test and the acidometric agar plate method.

- **Nitrocefin** can be used to screen for  $\beta$ -lactamase either as a reagent dropped onto colonies or in the form of a treated disk onto which colonies are rubbed. (This manual suggests using the disk method unless a laboratory is screening large numbers of isolates because the materials for the reagent tend to be available in bulk and costs can be high; methods for testing with the liquid nitrocefin reagent are included in the *N. gonorrhoeae* chapter [Chapter VI].)
  - a) Using sterile forceps or tweezers, place a nitrocefin disk on a clean slide; add a drop of distilled water.
  - b) Touch a sterile swab or loop to a characteristic colony from fresh, pure culture.
  - c) Rub the swab onto the moistened disk.
  - d) Observe the disk for five minutes; if the reaction is positive ( $\beta$ -lactamase producing strain), the areas of the disk containing growth will turn a characteristic red/pink color.
- A **modified acidometric agar plate method** is a differential agar method for testing *H. influenzae* isolates for the presence of  $\beta$ -lactamase activity [Park *et al.*

1978; Lucas 1979]. Penicillin and phenol red are combined in a non-nutrient plate; the pH indicator detects increased acidity resulting from the cleavage of the  $\beta$ -lactam ring of penicillin that yields penicilloic acid, and leads to a color change in the agar.

- a) Place a clump of isolated colonies in a discrete spot on the  $\beta$ -lactamase agar plate. Many strains can be tested on one plate; be certain to note their specific positions with proper labels.
- b) Apply known  $\beta$ -lactamase-positive and  $\beta$ -lactamase-negative control strains to the plate; label their positions.
- c) Incubate the plate in ambient air at 35°C for 15 minutes.
- d) Observe the plate for color change in the agar surrounding each discretely spaced colony. The agar surrounding positive-control strain should be yellow, whereas the agar surrounding the negative-control strain should not exhibit any change in color.

## Data for decision-making

Once the laboratory has assessed the serotype and antimicrobial susceptibility patterns of *H. influenzae* isolates, the information should be reported back to public health officials promptly. Factors to consider in the development of treatment policy include:

- Childhood immunizations should be considered if *H. influenzae* type b is a major local cause of invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors.



## *Neisseria meningitidis*

### CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

**N***eisseria meningitidis* is the etiologic agent of meningococcal disease, most commonly meningococcal bacteremia and meningitis. These two clinically overlapping syndromes may occur simultaneously, but meningitis alone occurs most frequently. *N. meningitidis* is an encapsulated bacterium and is classified into serogroups based on the immunological reactivity of the capsule's polysaccharide. The most common serogroups causing disease are A, B, C, Y, and W135. During the past 20 years, serogroups B and C have been responsible for most meningococcal disease in the Americas and Europe; serogroup A accounts for most meningococcal disease cases in Africa and some parts of Asia.

Meningococcal disease differs from the other leading causes of bacterial meningitis because of its potential to cause large-scale epidemics. Historically, these epidemics have been typically caused by serogroup A and, to a lesser extent, serogroup C. In Africa, the highest incidence rates of serogroup A meningococcal disease occur in a region of Sub-Saharan Africa extending from Sudan in the east to The Gambia in the west; this region consists of 15 countries comprised of more than 260 million people and has been referred to as the “meningitis belt.” During epidemics, children and young adults are most commonly affected, with attack rates as high as 1,000/100,000 population, or 100 times the rate of sporadic disease. The highest rates of endemic or sporadic disease occur in children less than 2 years of age. In recent years, two major epidemics of meningitis caused by *N. meningitidis* serogroup W135 have also been reported. In 2000, an outbreak of meningococcal disease in Saudi Arabia (which resulted in 253 cases and 70 deaths) was caused by a virulent clone of serogroup W135; this outbreak occurred simultaneously with the annual pilgrimage to Mecca and returning pilgrims disseminated this clone throughout the world, resulting in secondary cases. As of the time of writing of this laboratory manual in mid-2002, a serogroup W135 meningitis epidemic has been reported in Burkina Faso with more than 12,000 cases and 1400 deaths to date.

A quadrivalent polysaccharide vaccine that includes serogroups A, C, Y, and W135 is produced and used in the United States; however, bivalent A and C polysaccharide vaccines are being used in other parts of the world. New meningococcal conjugate vaccines are under development.

Laboratory personnel at risk for exposure to aerosolized *N. meningitidis* should ensure their protective vaccination status remains current and, if possible, work in a biological safety cabinet. Laboratory scientists who manipulate invasive *N. meningitidis* isolates in a manner that could induce aerosolization or droplet formation (including plating, subculturing, and serogrouping) on an open bench top and in the absence of effective protection from droplets or aerosols should consider antimicrobial chemoprophylaxis.

## Confirmatory identification of *N. meningitidis*

The following steps are recommended to confirm the identity of cultures that morphologically appear to be *N. meningitidis* (Figure 9). The best results are obtained with day-old cultures. Always check for purity of the growth by performing a Gram stain: *N. meningitidis* is a gram-negative, kidney-bean- or coffee-bean-shaped diplococcus (see Figure 72). When necessary, make subcultures to ensure purity. From growth on a blood agar plate, perform Kovac's oxidase test, and then identify the serogroup with a slide agglutination test. Finally, confirm the results with carbohydrate (*i.e.*, sugar) reactions.

Some laboratorians may be interested in the OMP (*i.e.*, outer membrane protein) subtyping of *N. meningitidis* isolates; these tests may be performed by international reference laboratories.

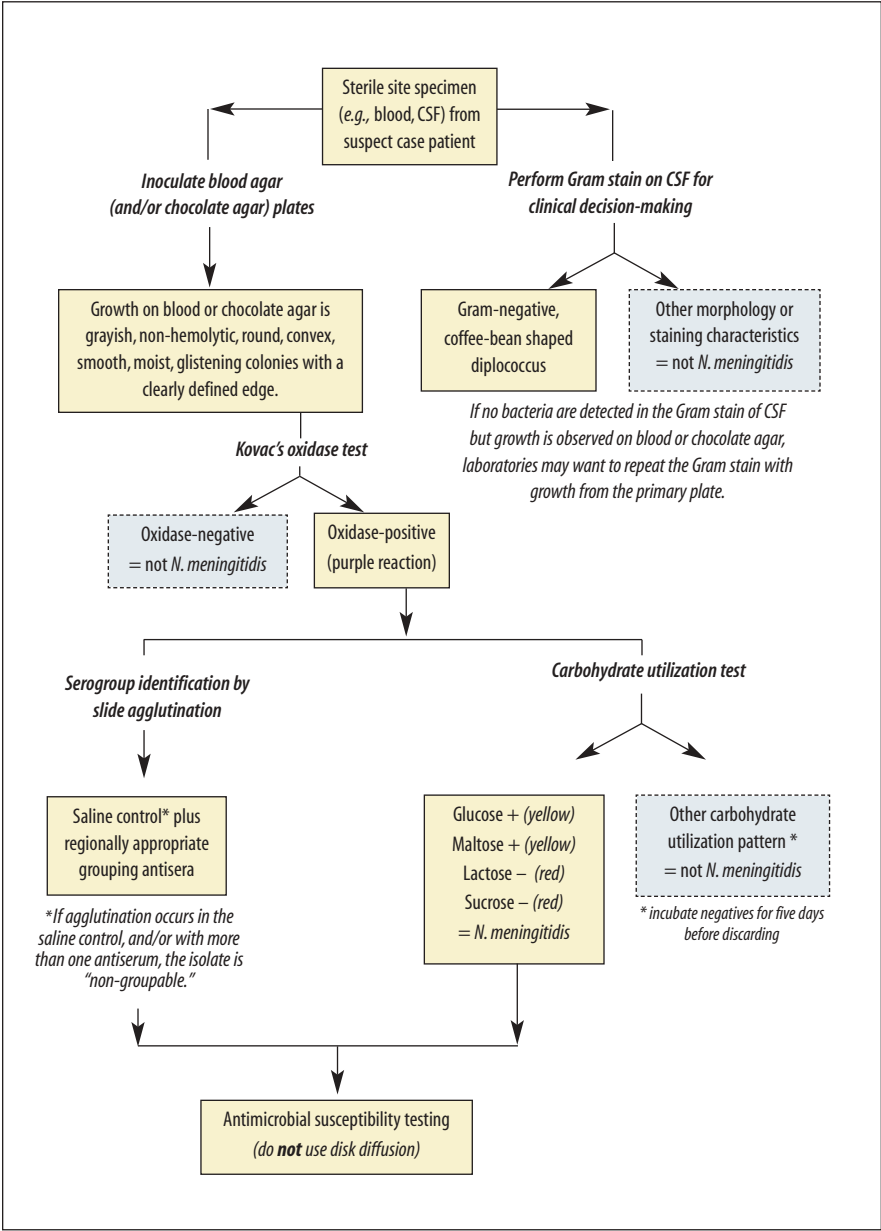
### Kovac's oxidase test for the identification of *N. meningitidis*

The oxidase test determines the presence of cytochrome oxidase. The Kovac's oxidase reagent (1% tetramethyl-*p*-phenylenediamine hydrochloride)<sup>7</sup> is turned into a purple compound by organisms containing cytochrome *c* as part of their respiratory chain; therefore, an oxidase-positive test will yield a purple reaction. (Instructions for making oxidase reagent are found in Appendix 2.)

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<sup>7</sup> 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**.

FIGURE 9: Flowchart for laboratory identification of *Neisseria meningitidis*



- a) Using a platinum inoculating loop, a disposable plastic loop, or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto a treated strip of filter paper (Figure 10). Do not use a Nichrome loop because it may produce a false-positive reaction.
- b) Positive reactions will develop within 10 seconds in the form of a purple color. Delayed reactions are unlikely with *N. meningitidis*.

The oxidase test aids in the recognition of *N. meningitidis* and other members of the genus *Neisseria*; other, unrelated, bacterial species with cytochrome c in the respiratory chain (e.g., *Pseudomonas aeruginosa* and *H. influenzae*) are also oxidase positive.

### Identification of the *N. meningitidis* serogroup

Twelve serogroups based on capsular polysaccharides are currently recognized: A, B, C, H, I, K, L, W135, X, Y, Z, and Z' (29E). (**Note:** serogroup D is no longer recognized.) Groups A and C are the common causes of meningitis outbreaks in Africa, but recently outbreaks caused by groups W135 and X have been reported; group B is a cause of endemic meningitis and may also cause outbreaks in some regions of the world (e.g., in Brazil). Grouping antisera are available commercially.

Serogrouping can be expensive, but it is valuable. Serogroup data provides laboratories and public health authorities with the tools to:

- identify outbreaks controllable by a vaccination campaign
- recognize the presence of serogroups causing sporadic disease
- detect the emergence of new outbreak strains (e.g., X or W135).

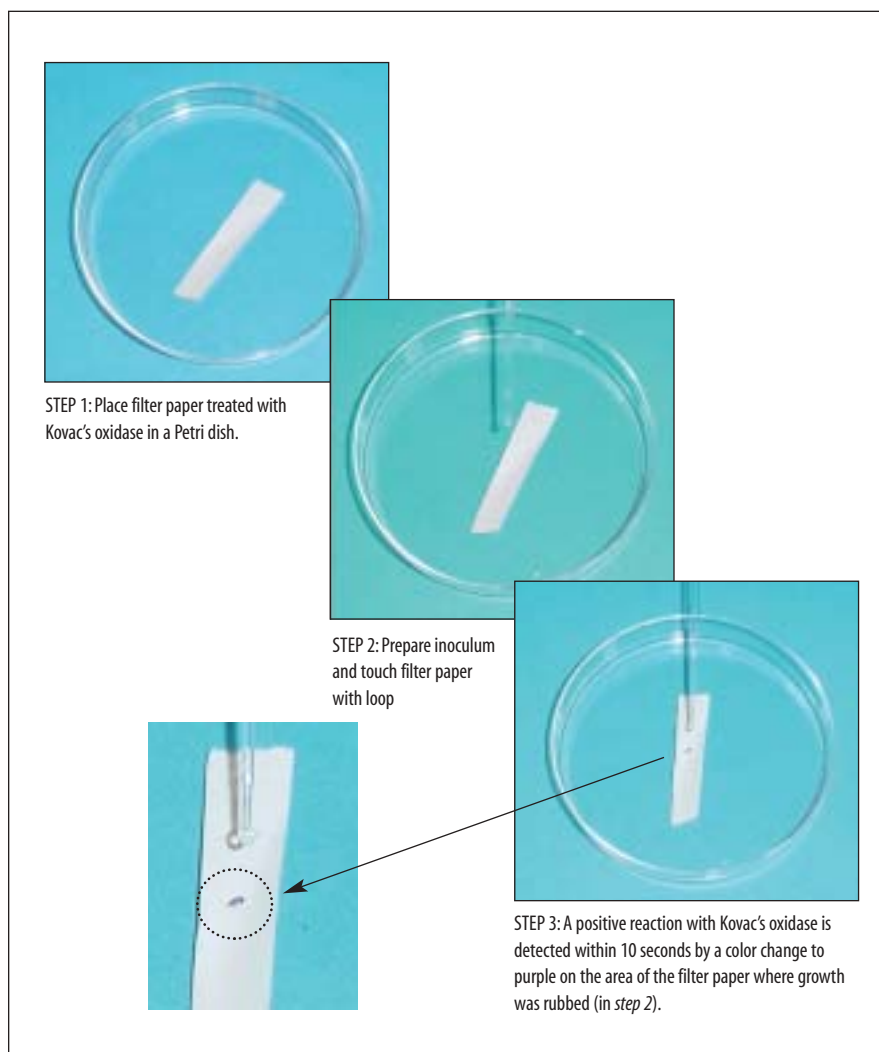
It is therefore essential that high-level reference laboratories have the capacity to isolate, identify and confirm the serogroup of *N. meningitidis* isolates causing sporadic disease as well as those they receive during the course of an outbreak.

### ***Slide agglutination test for serogrouping suspected *N. meningitidis* isolates***

The following methods require both formalinized physiological saline to make the meningococcal suspension and unformalinized physiological saline (or phosphate buffered saline [PBS]) to mix with the antisera. Store antisera in the refrigerator at 4°C when not in immediate use.

- a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slides into equal sections (e.g., three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.
- b) Collect a small portion of growth from the surface of an overnight culture on non-selective blood or chocolate agar plate using a sterile inoculating loop.

FIGURE 10: Kovac's oxidase test: a positive reaction on filter paper



Make a moderately milky suspension of the test culture in 250  $\mu$ l (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible. If working with only several isolates, it may be more convenient to make the suspension directly on the slide in 10  $\mu$ l of formalinized physiological saline per droplet.

- **Note:** For safety reasons, it is recommended that formalin-killed meningococcal suspensions rather than saline suspensions of living organisms be used; however, formalin is a carcinogen and must be stored

and handled with great care. (Alternatively, if formalin is not used to kill the meningococci, laboratorians can work under a safety hood.)

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a “moderately milky suspension” is roughly comparable to a 6 McFarland turbidity standard.
- c) Use a micropipettor or a bacteriologic loop to transfer a drop (5–10 µl) of the cell suspension to the lower portion of each section of the slide prepared in step a of this procedure.<sup>8</sup>
- d) Add a drop of group A antiserum above the drop of suspension in one of the test sections on the slide. In one of the other sections of the slide, add a drop of W135 antiserum below the drop of suspension in that section. For the third section of the slide, use the same method to add a drop of saline below the final drop of suspension.
- **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.
  - **Note:** In Africa, testing with A and W135 antisera (with a saline control to detect nonspecific autoagglutination) should be adequate for serologic characterization of most *N. meningitidis* isolates. Strains reacting negatively with A and W135 antisera should then be tested with other available antisera, particularly C, Y, B, and X.
- e) **Using a separate toothpick (or sterile loop) for each section**, mix each antiserum (and control saline) with its corresponding drop of cell suspension. Avoid contamination across the sections of the slide.
- f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, as it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the three mixed drops and read the slide agglutination reactions under bright light and over a black background (see Figure 2).
- g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figure 11 and Figure 42). When a strain reacts only in one grouping antiserum, it should be recorded as belonging to that serogroup.

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<sup>8</sup> This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only 5–10 µl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 µl of antisera are required for agglutination reactions to occur using the methods presented in this manual, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

(For example, an isolate exhibiting a strong agglutination reaction only in group A antiserum would be recorded as '*N. meningitidis*, serogroup A.')

- *If a strong agglutination reaction does not occur with the antisera tested:*
  - If the isolate is negative in the first two antisera tested (groups A and W135 in Africa) and the saline control, repeat the test with different antisera to identify the serogroup, following steps a through f of this procedure.
- *When a strain reacts with more than one antiserum or agglutinates in saline, the strain is categorized as non-groupable.* (These results occur rarely with fresh isolates, but they do happen occasionally.) Non-groupable results are characterized by:
  - 1) Autoagglutination in the saline control ("autoagglutinable").
  - 2) Cross-agglutination with reactions in more than one antiserum ("rough").
  - 3) No agglutination with either any of the antisera or with the saline control ("non-reactive").

Report results of *N. meningitidis* serogroup testing back to attending clinicians, as appropriate.

FIGURE 11: Positive and negative agglutination reactions on a slide: grouping antisera and saline control with *Neisseria meningitidis*



When a suspension is mixed with its homologous antiserum, agglutination occurs (*left*). In a negative reaction, as shown with a heterologous antiserum (*center*) or control saline (*right*), the suspension remains smooth and cloudy in appearance.

Carbohydrate utilization by *N. meningitidis*: cystine trypticase agar method

Carbohydrate utilization tests are used to further validate the identification of a strain as *N. meningitidis*. Various carbohydrates are added to the cystine trypticase agar (CTA) base to a final concentration of 1%. To confirm a culture as *N. meningitidis*, a set of four tubes, each containing a sugar (*i.e.*, glucose [dextrose], maltose, lactose, and sucrose) is used. Members of *Neisseria* species produce acid from carbohydrates by oxidation, not fermentation. *N. meningitidis* oxidizes glucose and maltose, but not lactose and sucrose. A phenol red indicator is included in the medium; it is a sensitive indicator that develops a yellow color in the presence of acid, at a pH of 6.8 or less. (Methods for the preparation and quality control of CTA medium are included in Appendix 2.)

- a) With an inoculating needle, collect a small amount of growth from an overnight culture of *N. meningitidis* on blood agar or chocolate agar.
- b) Stab the inoculum several times into the upper 10 mm of medium. Use another sterile needle, or flame the same needle, before inoculating each of the four carbohydrates to be tested.
- c) Fasten caps of tubes **tightly** and place in a 35°C incubator (without CO<sub>2</sub>). Incubate for at least 72 hours (and up to 5 days) before discarding as negative.
- d) Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth and the production of acid and is interpreted as a positive test (Figure 12). Although reactions may occur as early as 24 hours after inoculation, some reactions are delayed. If only glucose or maltose or none of the sugars react, continue incubation for up to 5 days before discarding. Occasionally, strains of *N. meningitidis* are encountered that utilize only dextrose or maltose but not both (Table 3).

TABLE 3: Carbohydrate utilization by some species of *Neisseria* and *Moraxella*

Species	Produces acid from <sup>a</sup>			
	Glucose <sup>b</sup>	Maltose	Lactose	Sucrose
<i>N. meningitidis</i>	+	+	–	–
<i>N. gonorrhoeae</i>	(+) <sup>c</sup>	–	–	–
<i>N. sicca</i>	+	+	–	+
<i>N. lactamica</i>	+	+	+	–
<i>M. catarrhalis</i>	–	–	–	–

<sup>a</sup> Negative results should not be interpreted prior to 72 hours of incubation in order to avoid false-negative results for delayed acid production reactions.

<sup>b</sup> Glucose may be also be referred to as “dextrose”.

<sup>c</sup> Strains of *N. gonorrhoeae* that are weak acid producers may appear to be glucose-negative in cystine trypticase agar (CTA) medium.

FIGURE 12: Cystine trypticase agar sugar reactions for acid production from carbohydrates by *Neisseria meningitidis*



Acid is produced by utilization of sugar and causes the CTA medium to turn yellow at or just below the agar surface. For *N. meningitidis*, there is utilization of dextrose and maltose (two tubes on left with yellow color just below the surface) and no utilization of lactose nor sucrose (two tubes on right with solid red color of medium).

### Commercial identification kits for *Neisseria*

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Neisseria* species. These systems may occasionally require supplemental tests, and other characteristics, such as microscopic and colony morphology, must be considered; additionally, antimicrobial susceptibility testing cannot be conducted without a confirmed *N. meningitidis* isolate. Generally, each system is self-contained, but addition of one or more reagents to complete certain reactions may be necessary. Follow the manufacturer's instructions precisely when using these kits. For detailed instructions and use of appropriate control strains, also consult the *Clinical Microbiology Procedures Handbook* (see Appendix 15). Rapid sugar utilization test kits may also be used toward the identification of *N. meningitidis*.

## Antimicrobial susceptibility testing of *N. meningitidis*

*N. meningitidis* does not commonly show resistance to many antimicrobial agents. Low-level resistance to penicillin is common in some areas of the world, though the clinical significance of this resistance has not yet been established.

Meningococcal resistance to sulfonamides, rifampicin (also referred to as rifampin), and chloramphenicol has also been described. Chloramphenicol tends to be the empiric drug of choice for treating patients with meningitis caused by *N. meningitidis*; rifampicin and sulfonamides are often used for prophylaxis.

**Antimicrobial susceptibility testing of *N. meningitidis* should not be performed by disk diffusion**, even though it is the least expensive screen, because results are very difficult to interpret and will not provide data useful for making informed treatment decisions. Two appropriate methods for testing include (1) minimal inhibitory concentration (MIC) determination by broth microdilution, and (2) use of the Etest® strip. The broth microdilution methodology provides laboratorians with quantitative MIC results based on the inhibition of growth of a standard inoculum in standard concentrations (dilutions) of antimicrobial. The Etest® antimicrobial susceptibility test methodology provides laboratorians with semi-quantitative MIC results, because although a standard suspension is used to inoculate a plate, the inoculum is not precisely standardized. Results of the Etest® and conventional MIC testing by broth microdilution are generally comparable.

The broth microdilution procedure can be expensive and challenging to perform and, because of the technical complexity required, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. For laboratories that do not perform MIC testing by dilution methods but still want to perform antimicrobial susceptibility tests on *N. meningitidis* isolates, the Etest® may be a convenient alternative.<sup>9</sup> The Etest® is easier to quality control and is the focus of this section of the manual, but broth microdilution methodology is included in Appendix 7. Figure 13 shows a sample worksheet for recording antimicrobial susceptibility test results for *N. meningitidis*.

Either 150-mm or 100-mm plates can be used for the Etest®, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate (see Figure 7).

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<sup>9</sup> The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

FIGURE 13. Sample form for recording antimicrobial susceptibility test results for *Neisseria meningitidis*

Date of Testing: ____/____/____		Interpretation of susceptibility: S = susceptible I = intermediate R = resistant				
Test performed by: _____						
Specimen number	Organism	Penicillin	Rifampicin (Rifampin)	Trimethoprim-sulfamethoxazole	Chloramphenicol	(other drug)
		<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>
		<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>
		<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>	<div>µg/ml</div> <div>S I R</div>
<i>S. pneumoniae</i> ATCC 49619 <sup>a</sup>	QC strain Q/C in range? →	<div>µg/ml</div> <div>Yes No</div>	<div>µg/ml</div> <div>Yes No</div>	<div>µg/ml</div> <div>Yes No</div>	<div>µg/ml</div> <div>Yes No</div>	<div>µg/ml</div> <div>Yes No</div>

<sup>a</sup> NCCLS has not validated MICs for *N. meningitidis*; this laboratory manual suggests using *S. pneumoniae* ATCC 49619 as the control strain.

[Source: Dr. F. Tenover, Centers for Disease Control and Prevention, Atlanta, GA USA: 2002.]

Received by: \_\_\_\_\_

Date of Report: \_\_\_\_/\_\_\_\_/\_\_\_\_

**Note:** After 18 – 22 hours of incubation, check the results for the quality control (QC) strain against the standard acceptable ranges; if they are in control, continue reading results for the test isolate. Record MIC results in µg/ml. (Breakpoints for interpretation of results may be found in Table 4.)

## Minimal inhibitory concentration testing of *N. meningitidis* by Etest® antimicrobial gradient strip

Mueller-Hinton + 5% sheep blood agar is used when testing *N. meningitidis* isolates with the Etest®. Follow the directions on the package insert included with the Etest® strips.

- a) Using a sterile cotton-tip applicator, touch the surface of one to four morphologically similar, isolated colonies grown on a chocolate agar plate incubated in CO<sub>2</sub>-enhanced atmosphere (5% CO<sub>2</sub> in a CO<sub>2</sub>-incubator or candle-extinction jar) at 35°C for 18–22 hours. Immerse the applicator into a tube containing sterile broth (e.g., Muller-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 the cells to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**
- b) Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard. If the turbidity of the inoculum is greater than the standard, dilute it with broth to equal the turbidity of the standard. (Figures 51 and 52 in Appendix 2 show how to compare the turbidity of the suspension with the standard and also provide black and white lines as a reading background.)
- c) Immerse a sterile cotton-tipped swab into the adjusted inoculum (prepared in step *b* of this procedure). Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a 15x150-mm Mueller-Hinton + 5% sheep blood agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- d) Allow the inoculum to dry on the surface of the plate (which should take approximately 10 minutes). **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20°C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the antimicrobial gradient strips that will not be used in this batch of testing to the -20°C freezer.
- e) When the surface of the inoculated plate is dry and the Etest® strips are at room temperature, place the antimicrobial gradient strips onto the agar with an Etest® applicator or sterile forceps, as illustrated in Figure 7. Make sure that the printed MIC values are facing upward (i.e., that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) **Once applied, it is important to not move the antimicrobial gradient strips.**

- f) Incubate the plates in an inverted position in a 5% CO<sub>2</sub> atmosphere for 18–22 hours at 35°C. A candle-extinction jar may be used if a CO<sub>2</sub>-incubator is not available. Because *N. meningitidis* grows 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.

After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.** MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,<sup>10</sup> and shows drug-related effects, technical and handling effects, organism-related effects, and resistance mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of an isolate to penicillin, an MIC recorded from the graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml.

### **Quality control for antimicrobial susceptibility testing of *N. meningitidis***

To verify that antimicrobial susceptibility test results are accurate, it is important to include at least one control organism. It is of note here that NCCLS<sup>11</sup> does not publish MIC ranges specific to *N. meningitidis*; however, the Centers for Disease Control and Prevention (CDC, United States of America) recommends that if antimicrobial susceptibility testing of *N. meningitidis* is going to be performed, then a banked control strain for fastidious organisms (*S. pneumoniae* ATCC 49619) should be used for quality control. The NCCLS MIC ranges for quality control testing of *S. pneumoniae* ATCC 49619 with the antimicrobial agents penicillin, rifampicin, and sulfonamides are included in Table 4. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

<sup>10</sup> AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abbiobisk.com>.

<sup>11</sup> Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

TABLE 4: Minimal inhibitory concentration (MIC) ranges for quality control of *Neisseria meningitidis* antimicrobial susceptibility testing

QC strain for <i>N. meningitidis</i> <sup>a</sup>	Penicillin MIC Range <sup>b</sup>	Rifampicin (Rifampin) MIC Range <sup>b</sup>	Trimethoprim-sulfamethoxazole MIC Range <sup>b</sup>	Chloramphenicol MIC Range <sup>b</sup>
<i>S. pneumoniae</i> ATCC 49619	0.25 – 1 µg/ml	0.015 – 0.06 µg/ml	0.12/2.4 – 1/19 µg/ml	2 – 8 µg/ml

<sup>a</sup> Source: Dr. F. Tenover, Centers for Disease Control and Prevention, Atlanta, Georgia, USA: 2002.

<sup>b</sup> Source: NCCLS (2002) *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, PA 19087 USA.

Resistance to antimicrobials other than penicillins and rifampicin is not commonly detected in *N. meningitidis*; however, laboratories, clinicians, and other public health practitioners may be interested in performing annual screens of isolates in storage. (Appendix 11 provides methods for how to preserve and store meningococcal isolates.) Periodic, non-routine surveillance for characteristics such as  $\beta$ -lactamase production, and ceftriaxone, chloramphenicol and fluoroquinolone resistance will help provide information to public health agencies and international reference laboratories regarding the emergence of new *N. meningitidis* strains of clinical and public health concern.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct for trimethoprim-sulfamethoxazole (cotrimoxazole); organisms may then appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm or the pH is not between 7.2 and 7.4, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected. **(Do not attempt to adjust the pH of this Mueller-Hinton agar even if it is out of range; see Appendix 2)**

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even when the isolates being tested are susceptible.

*Reading and interpreting the Etests®*

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip, as illustrated in Figure 8. Record the quality control results first. If zones

produced by the control strain are out of the expected ranges (Table 4), the laboratorian should consider possible sources of error. If all antimicrobial agents are in control, read the test MICs. Note any trailing endpoints.

Because antimicrobial susceptibility test results can be affected by many factors not necessarily associated with the actual susceptibility of the organism (e.g., inoculum size, agar depth, storage, time, and others), quality control practices must be followed carefully.

Although NCCLS has not defined standardized breakpoints for the interpretation of an *N. meningitidis* isolate as susceptible or not, the MICs obtained by antimicrobial susceptibility testing methods as described in this document can still be used. Just as a laboratory might assess antimicrobial susceptibility for the very many other organisms for which no NCCLS breakpoints have been defined, laboratorians and clinicians should consider the site of infection in conjunction with the dose and pharmacokinetics of the antimicrobial agent to determine how much drug reaches the site of the infection. This information should then be compared to the MIC value to determine if the concentration of drug available is at least four times greater than the MIC. If the concentration of drug available is  $\geq 4$  times the MIC, the organism may be considered susceptible; if not, it is resistant.

## Data for decision-making

Once the laboratory has confirmed the identification and serogroup (and antimicrobial susceptibility patterns, if appropriate) of *N. meningitidis* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- Immunization should be considered if a *N. meningitidis* vaccine serotype is a major cause of local invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors.



## *Streptococcus pneumoniae*

### CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

*Streptococcus pneumoniae* is a common agent of lower and upper respiratory diseases, such as pneumonia, meningitis and acute otitis media (middle ear infections), affecting children and adults worldwide. This bacterial pathogen is the cause of approximately 40% of acute otitis media. Although acute otitis media and other upper respiratory tract infections do not commonly progress to invasive disease they do contribute significantly to the burden and cost of pneumococcal disease. Meningitis in infants, young children and the elderly is often caused by *S. pneumoniae*. Persons who have sickle cell disease, anatomic asplenia, or are immunocompromised also have increased susceptibility to *S. pneumoniae* infection. Pneumococcal meningitis is the most severe presentation of disease, but most illnesses and deaths result from pneumococcal pneumonia. Pneumococcal polysaccharide vaccine has been available for preventing invasive disease in the elderly and in persons with chronic illnesses that may impair their natural immunity to pneumococcal disease; however, this vaccine is not effective in children <2 years of age. In contrast to polysaccharide vaccines, conjugate vaccines are effective in young children. A pneumococcal conjugate vaccine covering seven serotypes that most commonly cause bacteremia in children in the United States (and some other industrialized nations) was approved for clinical use in 2000; research on vaccine formulations containing serotypes more common in developing countries is underway.

*S. pneumoniae* is frequently carried in the throat without causing disease. On occasion, public health investigations call for studies on the prevalence of *S. pneumoniae* carriage. For this research, samples may be collected using nasopharyngeal (NP) swabs; methodology for collection and isolation with NP swabs is included in Appendix 5. Antimicrobial susceptibility testing on isolates should be performed as presented in this chapter.

## Confirmatory identification of *S. pneumoniae*

*S. pneumoniae* are gram-positive diplococci or chains of cocci (see Figure 73). On blood agar and chocolate agar plates, *S. pneumoniae* colonies appear small, greyish and mucoid (*i.e.*, watery), and are surrounded by a greenish zone of alpha-hemolysis ( $\alpha$ -hemolysis).

Colonies of pneumococci and  $\alpha$ -hemolytic viridans streptococci each appear raised when young; however, after 24–48 hours, the center of pneumococcal colonies becomes depressed, whereas viridans streptococcal colonies retain their raised appearance (Figure 14). A 3x hand-lens or a microscope (30x–50x) can therefore be a useful aid in differentiating pneumococci from  $\alpha$ -hemolytic viridans streptococci. Laboratory differentiation between *S. pneumoniae* and viridans streptococci is accomplished by optochin and bile solubility testing: pneumococci are susceptible to optochin and bile-soluble, while viridans streptococci are not. Commercially available slide agglutination tests can also be used for identification of pneumococci. For optimal results plates for pneumococcal identification assays should be incubated in a 5% CO<sub>2</sub> atmosphere.

A flowchart diagram of the laboratory identification of *S. pneumoniae* is included in Figure 15. Presumptive identification of *S. pneumoniae* is made by determining the susceptibility of the strain to optochin (*i.e.*, ethylhydrocupreine). The bile solubility test is also used for identification of *S. pneumoniae*, particularly when results of the optochin susceptibility test are ambiguous.

### Optochin susceptibility test

The optochin susceptibility test is performed with a 6-mm, 5- $\mu$ g optochin disk,<sup>12</sup> and is used to differentiate between *S. pneumoniae* and viridans streptococci. Optochin-susceptible strains can be identified as *S. pneumoniae*.

### Performance of the optochin susceptibility test

- a) Touch the suspect  $\alpha$ -hemolytic colony with a sterile bacteriological loop and streak for isolation onto a blood agar plate in a straight line. Several strains can be tested on the same plate at once, streaked in parallel lines and properly labeled.
- b) Aseptically place an optochin or “P” disk with a diameter of 6 mm (and containing 5  $\mu$ g of ethylhydrocupreine) on the streak of inoculum, near the end where the wire loop was first placed. Because the inoculum is streaked in a straight line, three to four colonies may be tested on the same plate (Figure 16).

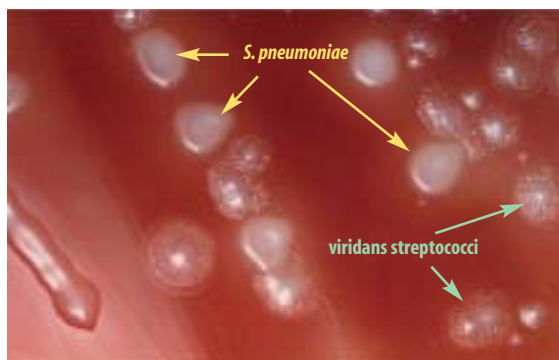
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<sup>12</sup> The results and interpretation of the optochin susceptibility test presented in this document are appropriate for the 6-mm, 5- $\mu$ g optochin disk (“P”-disk), although different size disks (and possibly optochin concentrations) are available for purchase. When using optochin disks with different size and/or concentration parameters, follow the manufacturer’s instructions for interpretation.

FIGURE 14: A properly streaked blood agar plate with pneumococci and viridans streptococci



Note how growth is heavy where streaking began on the left and then thins to individual colonies.



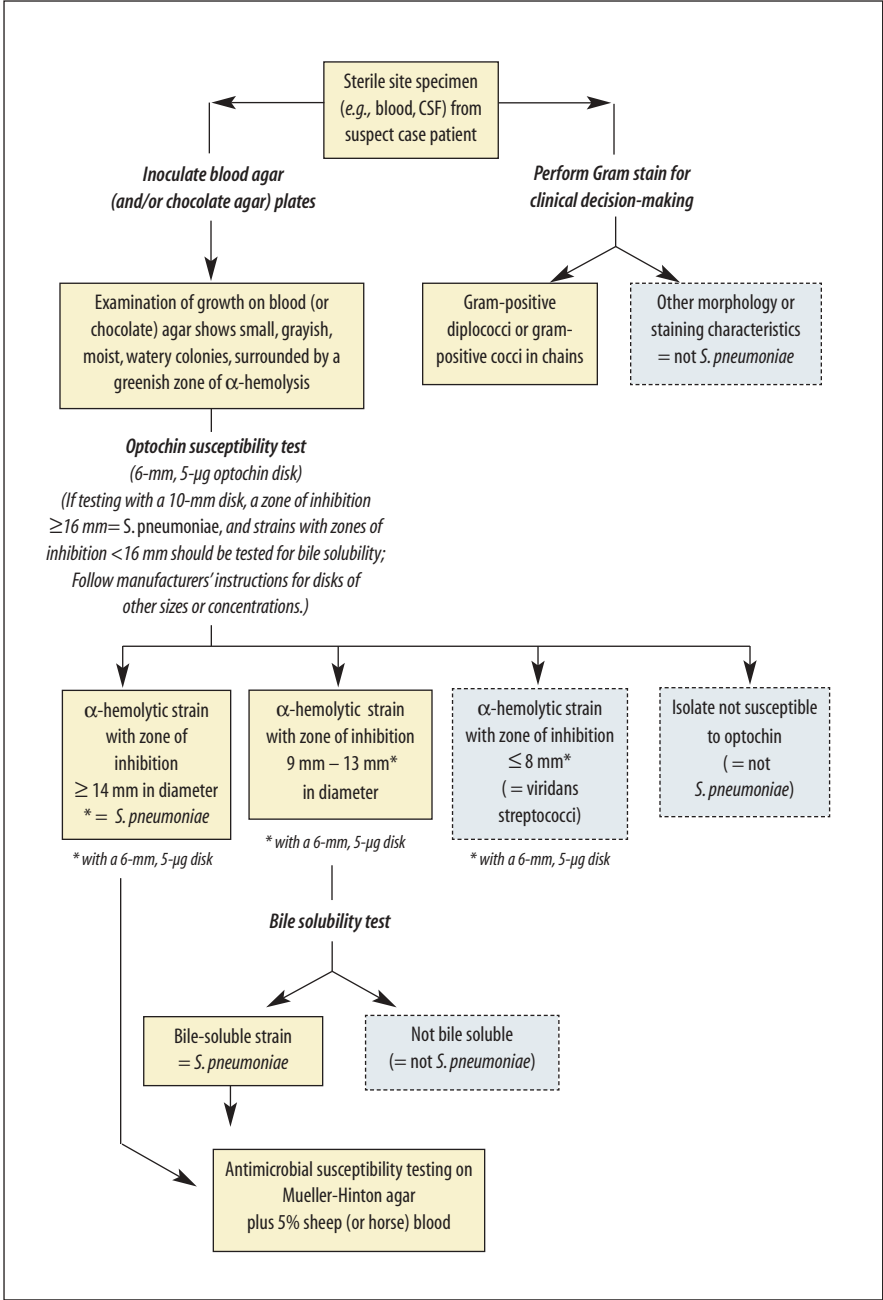
The *S. pneumoniae* has a depressed center (yellow arrows) at 24 – 48 hours incubation, whereas the viridans streptococci retain a raised center (black arrows).

- c) Incubate the plates in a CO<sub>2</sub>-incubator or candle-jar at 35°C for 18–24 hours.
- d) Read, record, and interpret the results.

#### ***Reading and interpreting the optochin susceptibility test results***

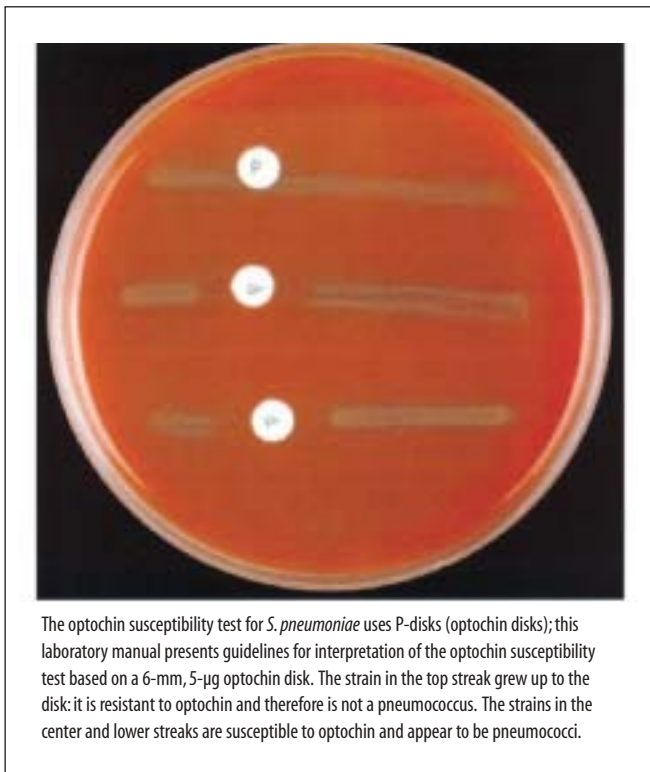
In Figure 16, the strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the center and lower streaks are susceptible to optochin and appear to be pneumococci.

FIGURE 15: Flowchart for laboratory identification of *Streptococcus pneumoniae*



- $\alpha$ -hemolytic strains with a zone of inhibition of growth greater than 14 mm in diameter are pneumococci.  
(If using a 10-mm, 5- $\mu$ g disk,  $\alpha$ -hemolytic isolates with a zone of inhibition of growth >16 mm in diameter are considered susceptible to optochin and, therefore, are pneumococci.)
- $\alpha$ -hemolytic strains with no zones of inhibition are viridans streptococci.
- **$\alpha$ -hemolytic strains with zones of inhibition ranging between 9 mm and 13 mm should be tested for bile solubility** for further characterization and identification.  
(If using a 10-mm disk,  $\alpha$ -hemolytic isolates with a zone of inhibition of growth <16 mm should be tested for bile solubility.)

FIGURE 16: Optochin susceptibility test for identification of *Streptococcus pneumoniae*



## Bile solubility test

The bile solubility test is performed on isolates with small zones of inhibition in the optochin susceptibility test. It can be performed using either the “tube method” or the “plate method.”

### *Tube method for the performance of the bile solubility test*

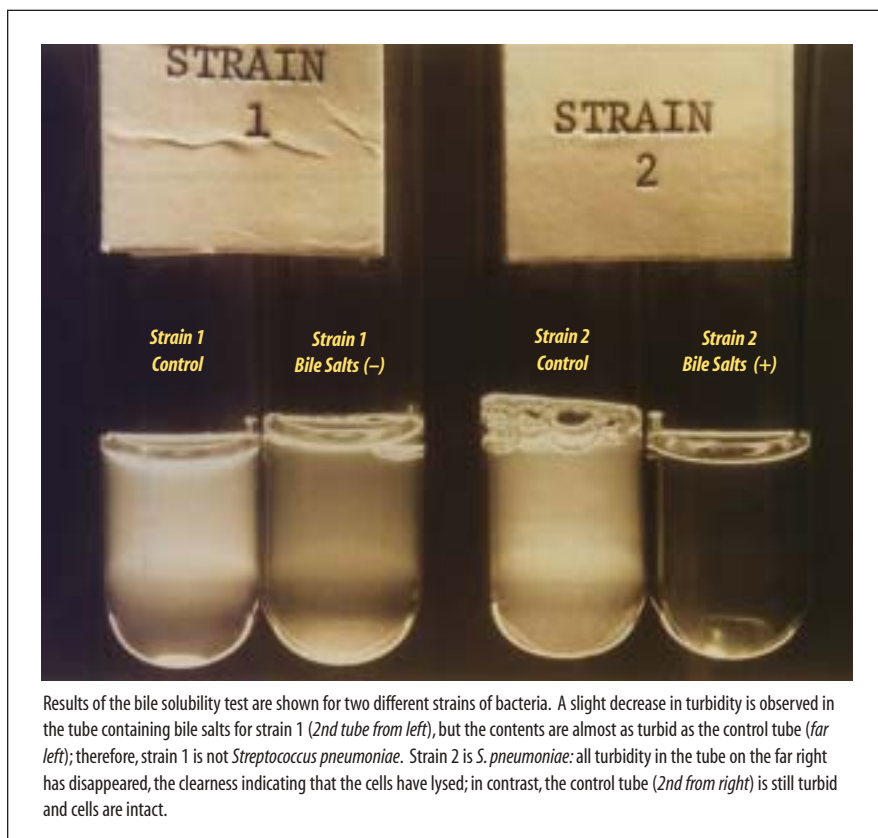
Two tubes are required for bile solubility testing of each suspect strain of *S. pneumoniae*.

- a) Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a bacterial cell suspension in 0.5 ml of sterile saline. The suspension of bacterial cells should be cloudy, similar to that of a 0.5 or 1.0 McFarland turbidity standard. (Preparation of a McFarland turbidity standard is described in Appendix 2.)
  - If growth on the optochin test plate is sufficient, the suspension can be made with the bacterial cells collected from the specific streak of suspect *S. pneumoniae*.
  - When there is insufficient growth to make a suspension of the proper density in 0.5 ml of sterile saline, inoculate a blood agar plate with the suspect growth and incubate overnight (*i.e.*, for 18 – 24 hours at 35°C in a CO<sub>2</sub>-enriched atmosphere) to prepare a fresh culture.
- b) Divide the suspension into two equal amounts (*i.e.*, 0.25 ml per tube). Add 0.25 ml of saline to one tube and 0.25 ml of 2% sodium desoxycholate (bile salts) to the other.
  - To make a 2% concentration of bile salts, add 0.2 g of sodium desoxycholate to 10 ml of saline.
- c) Shake the tubes gently and incubate them at 35° – 37°C for up to 2 hours.
- d) Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result (Figure 17).
  - Strains that yield clearing of the suspension in tube in the bile solubility test should be reported as “bile soluble.”
  - Strains for which the turbidity in the tube remains the same as that in the saline control tube is reported as negative for bile solubility (or “bile insoluble” or “bile resistant”).

### *Plate method for the performance of the bile solubility test*

In place of the tube test for bile insolubility, a laboratorian may perform the bile solubility test using the plate method. **A freshly prepared culture** of the suspect organism must be used for this test.

FIGURE 17: Positive and negative results of the bile solubility test



- a) Place a drop of 10% sodium desoxycholate solution directly on a colony of the suspect pneumococcal strain to be tested.
  - (To prepare the 10% solution of bile salts, add 1 g of sodium desoxycholate bile salts to 10 ml of sterile saline.)
- b) Keep the plate at room temperature (*i.e.*, 72°–75°C) or place it face up (*i.e.*, agar-side up) and on a level surface in an ambient air incubator (*i.e.*, **not** a CO<sub>2</sub>-incubator) at 35°C for approximately 15 minutes (or until the 10% bile salt reagent dries).
  - *Optional:* instead of leaving the plate out at room temperature, laboratorians may choose to put the plate top-side (*i.e.*, agar-side) up on a level surface in an ambient air incubator (*i.e.*, **not** a CO<sub>2</sub>-incubator) at 35°C until the reagent dries (approximately 10–15 minutes).
- c) When the reagent dropped on the suspect colony is dry, read, record, and interpret the results.

Pneumococcal colonies are bile-soluble and will disappear or appear as flattened colonies; in contrast, bile-resistant streptococcal colonies will be unaffected.

### Interpretation of the combined optochin and bile solubility tests for pneumococcal identification

The following summary of results of the optochin and bile-solubility tests is commonly used to accurately and conveniently identify *S. pneumoniae* (i.e., pneumococcus).

- A strain exhibiting a zone of inhibition by optochin  $\geq 14$  mm (with a 6-mm, 5- $\mu$ g disk) is a pneumococcus.
- A strain exhibiting a smaller but definite zone of inhibition by optochin (9–13 mm with a 6-mm, 5-mg disk) **and** that is also bile soluble is a pneumococcus.

The following summary of results of the optochin and bile-solubility tests should be interpreted as negative for *S. pneumoniae* (and positive for viridans streptococci).

- A strain with a small zone of inhibition by optochin ( $\leq 8$  mm with a 6-mm, 5- $\mu$ g disk) that is not bile soluble is not a pneumococcus. (The colonies can be identified as viridans streptococci.)
- Strains with no zones of inhibition by optochin are not pneumococci. (The colonies can be identified as viridans streptococci.)

### Commercial test kits for identification (slide agglutination test)

Commercially available slide agglutination tests (e.g., Slidex Pneumo-kit® and the Pneumoslide™) can also help identify colony growth from blood agar plates as *S. pneumoniae*. Follow the manufacturer's instructions precisely when using these and any other commercial tests.

If a colony appears to be *S. pneumoniae* on the basis of morphology and susceptibility to optochin, but it has a negative bile solubility test, slide agglutination tests can assist with identification of the isolate. A positive slide agglutination test should be interpreted as a possible *S. pneumoniae* isolate, whereas a negative slide agglutination reaction in conjunction with the positive optochin and negative bile solubility would indicate the isolate is not *S. pneumoniae*.

### Identification of the *S. pneumoniae* serotype

Serotyping of pneumococci is not usually necessary for a clinical response. However, in some situations or settings (e.g., studies focusing on evaluation

of vaccine efficacy), it will be appropriate to type these isolates. Methods for serotyping and Quellung typing are included in Appendix 6.

## Antimicrobial susceptibility testing of *S. pneumoniae*

The results of antimicrobial susceptibility tests will be used to help make recommendations for clinical treatment. There are a variety of methods by which one can determine the antimicrobial susceptibility of a bacterial pathogen, commonly including disk diffusion, testing by agar dilution or broth microdilution, and testing by antimicrobial gradient agar diffusion (e.g., with the Etest® strip). The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;<sup>13</sup> if performed precisely according to the following protocol, this method will yield data that can reliably predict the *in vivo* effectiveness of the drug in question. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results for *S. pneumoniae*.

The disk diffusion method gives valid data for only certain antibiotics, so this laboratory manual recommends use of the Etest® to gather data about the minimal inhibitory concentration (MIC) of antimicrobial agents.<sup>14</sup> MIC testing can also be done by dilution; however because agar dilution and broth microdilution are expensive and technically complex, this manual recommends that countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. (Alternatively, if resources are available, laboratories may purchase commercially-available, frozen MIC panels and follow the manufacturer's instructions to carry out the MIC test.)

This laboratory manual describes antimicrobial susceptibility testing of *S. pneumoniae* by the disk diffusion method and by the Etest® antimicrobial gradient strip method. (Figure 18 is a sample worksheet for recording results of the antimicrobial susceptibility tests.) Although disk diffusion will provide information for most antimicrobial agents regarding interpretation of a strain as susceptible, intermediate, or resistant, the Etest® provides general information about the MIC of antibiotic. **The accuracy and reproducibility of this test are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis.**

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<sup>13</sup> Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

<sup>14</sup> The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

FIGURE 18: Sample form for recording antimicrobial susceptibility test results for *Streptococcus pneumoniae*

Date of Testing: ____/____/____ Test performed by: _____						
Interpretation of susceptibility: S = susceptible I = intermediate R = resistant						
Specimen number	Meningitis isolate? <sup>a</sup>	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Oxacillin <sup>b</sup> (disk) or (Penicillin) (MIC)	(other drug)
			mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R
			mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R
			mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R	mm µg/ml  S I R
ATCC 49619	N/A	NCCLS QC strain QC in range? →	mm µg/ml  Yes No	mm µg/ml  Yes No	mm µg/ml  Yes No	mm µg/ml  Yes No

<sup>a</sup> if an *S. pneumoniae* isolate is from a meningitis patient, the breakpoints for interpretation of the MIC may differ from those for isolates from other sites.  
<sup>b</sup> if an oxacillin disk yields a zone diameter <20 mm for *S. pneumoniae*, MIC testing to a specific penicillin must be done in order to interpret the susceptibility.

Reviewed by: \_\_\_\_\_ Date of Report: \_\_\_\_/\_\_\_\_/\_\_\_\_

**Note:** After 20-24 hours of incubation, check the results for the quality control (QC) strains against the standard acceptable ranges; if they are within control limits, continue reading results for the test isolate. Record disk diffusion results in mm and MIC results in µg/ml. (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 5.)

## Quality control of antimicrobial susceptibility testing of *S. pneumoniae*

Quality control tests must be performed as part of the normal laboratory routine. To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test or new set of testing conditions. *S. pneumoniae* ATCC 49619 is the NCCLS control strain to use when performing antimicrobial susceptibility testing on *S. pneumoniae* isolates.

**Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 5.** If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

- **Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other environmental factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not.
- **If the depth of the agar in the plate is not uniformly 3–4 mm,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the pH of the test medium is not between 7.2 and 7.4,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected. (**Note:** Do not attempt to adjust the pH of the Mueller-Hinton agar test medium if it is outside the range; see Appendix 2.)
- **If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, even if the isolates are susceptible, when colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks.

Quality control (“QC”) tests should be performed once per week if antimicrobial susceptibility tests are performed daily after 30 days of in-control results, or with every group of tests when testing is done less frequently. They should also be done with each new batch of antimicrobial susceptibility test medium and every time a new lot of disks is used.

## Antimicrobial susceptibility testing by disk diffusion

Antimicrobial susceptibility can be determined using the disk diffusion method; however, disk diffusion antimicrobial susceptibility testing is generally not performed on meningitis isolates. This laboratory manual describes the optimal media, inoculum, antimicrobial agents to be tested, incubation conditions, and interpretation of results.

- Mueller-Hinton agar medium supplemented with 5% sheep blood is recommended for determining the antimicrobial susceptibility of *S. pneumoniae* specimens by disk diffusion. The agar plates should have a uniform depth of 3–4 mm.
- The 1- $\mu$ g oxacillin disk is recommended for predicting the susceptibility of *S. pneumoniae* to penicillin because penicillin disks do not provide reproducible results. Interpretations of the oxacillin disk diffusion test are generalizable across the  $\beta$ -lactam drugs for *S. pneumoniae*.
  - **It is only possible to conclude if a strain is susceptible to penicillin based on the oxacillin screen, and not if it is resistant to penicillin. If the zone of inhibition around the oxacillin disk is less than 20 mm, additional MIC testing (e.g., by Etest®) must be performed to assess whether the isolate is resistant or susceptible to penicillin.**
- A 30- $\mu$ g chloramphenicol disk is used for detecting resistance to chloramphenicol.
- A 25- $\mu$ g trimethoprim-sulfamethoxazole (cotrimoxazole) disk (i.e., a disk comprised of 1.25 mg trimethoprim plus 23.75 mg sulfamethoxazole) is used for detecting trimethoprim-sulfamethoxazole resistance in *S. pneumoniae*. **The Mueller-Hinton agar used for this test should be thymidine free to obtain accurate results with trimethoprim-sulfamethoxazole.**

### *Methods for antimicrobial susceptibility testing by disk diffusion*

Prepare the inoculum for antimicrobial susceptibility testing of *S. pneumoniae* from fresh pure cultures of *S. pneumoniae* (grown overnight on blood or chocolate agar). Prepare cell suspensions of the bacteria to be tested in sterile physiological saline or Mueller-Hinton broth. A cell suspension equal to a density of a 0.5 McFarland turbidity standard is used for the inoculum. (Preparation of a McFarland turbidity standard and plate count methods are described in Appendix 2.)

- a) Suspend viable colonies from an overnight sheep blood or chocolate agar plate in a tube of broth to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells with the broth. **This suspension should be used within 15 minutes.**

- b) Compare the density of the suspension to the 0.5 McFarland turbidity standard by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines (see Figures 51 and 52, Appendix 2). If the density is too heavy, the suspension should be diluted with additional suspending medium (*i.e.*, saline or broth). If the density is too light, additional bacteria should be added to the suspension.
- c) When the proper density is achieved, dip a cotton or dacron swab into the bacterial suspension. Lift it out of the broth and remove excess fluid by pressing and rotating the swab against the wall of the tube.
- d) Use the swab to inoculate the entire surface of the supplemented Mueller-Hinton agar plate three times, rotating the plate 60 degrees between each inoculation (see Figure 34). Use the same swab with each rotated streak, but **do not re-dip the swab in the inoculum** (*i.e.*, the bacterial cell suspension).
- e) Allow the inoculum to dry before placing the disks on the plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. (If drying takes longer than 15 minutes, use a smaller volume of inoculum in the future.)
- f) After the plate is dry, place the antimicrobial disks on the plates (as shown in Figure 6). Use sterile forceps to place the disks on the Mueller Hinton agar and tap them gently to ensure they adhere to the agar. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**
- g) Incubate the plates in an inverted position in a 5% CO<sub>2</sub> atmosphere for 20–24 hours at 35°C. A candle-extinction jar may be used if a CO<sub>2</sub>-incubator is not available.
  - If this is a new batch of Mueller-Hinton agar, the antimicrobial disks are new, or it is an otherwise appropriate time to perform quality control, follow steps a through g above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference QC strains are included in Table 5.
- h) After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (see Figure 6).
  - Care should be taken not to touch the disk or surface of the agar. Sterilize the ruler occasionally to prevent transmission of the bacteria. In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony. Record the results in millimeters (mm). Figure 5 provides a sample form for recording results.

TABLE 5: Antimicrobial susceptibility test breakpoints and quality control ranges for *Streptococcus pneumoniae*

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml) <sup>a</sup>			NCCLS QC strain <i>S. pneumoniae</i> ATCC 49619
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	≥ 21 mm (≤ 4 µg/ml)	~ ~	≤ 20 mm (≥ 8 µg/ml)	23 – 27 mm (2 – 8 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 19 mm (≤ 0.5 – 9.5 µg/ml)	16 – 18 mm (1/19 – 2/38 µg/ml)	≤ 15 mm (≥ 4/76 µg/ml)	20 – 28 mm (0.12/2.4 – 1/19 µg/ml)
Oxacillin <sup>b</sup>	1 µg Disk diffusion ONLY	≥ 20 mm <sup>b</sup>	** <sup>b</sup>	** <sup>b</sup>	≤ 12mm <sup>c</sup>
Penicillin <sup>b</sup>	MIC ONLY	(≤ 0.06 µg/ml)	(0.12 – 1 µg/ml)	(≥ 2 µg/ml)	(0.25 – 1 µg/ml)
Ceftriaxone <sup>d,e</sup>	MIC ONLY				
	Non-meningitis isolate MIC	(≤ 1 µg/ml)	(2 µg/ml)	(≥ 4 µg/ml)	(0.03 – 0.12 µg/ml)
	Meningitis isolate MIC	(≤ 0.5 µg/ml)	(1 µg/ml)	(≥ 2 µg/ml)	
Cefotaxime <sup>d,e</sup>	MIC ONLY				
	Non-meningitis isolate MIC	(≤ 1 µg/ml)	(2 µg/ml)	(≥ 4 µg/ml)	(0.03 – 0.12 µg/ml)
	Meningitis isolate MIC	(≤ 0.5 µg/ml)	(1 µg/ml)	(≥ 2 µg/ml)	

<sup>a</sup> Source: NCCLS (2002) *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, PA 19087-1898 USA.

<sup>b</sup> Oxacillin should only be tested by disk diffusion. If the zone is <20 mm, an isolate cannot be reported as susceptible, intermediate or resistant, and MIC testing must be conducted for an appropriate penicillin (or other β-lactam) drug.

<sup>c</sup> "Deterioration in oxacillin disk content is best assessed with QC organism *Staphylococcus aureus* ATCC 25923, with an acceptable zone diameter of 18 to 24 mm" [NCCLS 2002].

<sup>d</sup> Penicillin, ceftriaxone, and cefotaxime should only be tested by a method that will provide an MIC; it is presented in this table as a follow-up for an equivocal oxacillin disk diffusion test (i.e., oxacillin zone of inhibition <20 mm). Perform MIC testing on the specific penicillin (or other β-lactam) drug that would be used to treat.

<sup>e</sup> Ceftriaxone and cefotaxime have separate interpretive MIC breakpoints for meningitis and non-meningitis isolates.

- i) Interpret the antimicrobial susceptibility of the test strain (and check that results for the QC strain *S. pneumoniae* ATCC 49619 are within the acceptable control range) by comparing the results to the NCCLS standard zone sizes (Table 5).

### The Etest® for minimal inhibitory concentration testing of *S. pneumoniae*

For *S. pneumoniae*, disk diffusion testing indicates whether an organism is susceptible or resistant to an antimicrobial for most agents. However, disk diffusion testing for pneumococcal isolates and oxacillin (a penicillin agent) is not sufficient to distinguish between complete and intermediate resistance. For surveillance purposes, a laboratory may want to quantify the results of the oxacillin

disk diffusion test by performing minimal inhibitory concentration (MIC) testing of penicillin or any other beta-lactam antibiotic that would be used for treatment. As mentioned earlier in this manual, MIC testing by dilution can be expensive and challenging, and because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. The World Health Organization (WHO) recommends that only one laboratory in a resource-limited region perform antimicrobial susceptibility testing; however, in countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted at each laboratory in accordance with the standardized guidelines presented in this manual.

Laboratorians determining the minimal inhibitory concentration (MIC) for resistant isolates must be highly skilled in performing these tests and committed to obtaining accurate and reproducible results. In addition, a national (or regional) reference laboratory must have the ability and resources to store isolates either by lyophilization or by freezing at -70°C. Methods for preservation and storage of isolates are presented in Appendix 11, and detailed methods for transport of isolates according to international regulations are presented in Appendix 12.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest® serves as a test method that is both convenient and reliable.<sup>15</sup> The Etest® requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest® strips must be consistently stored in a freezer at -20°C.**

The Etest® is an antimicrobial susceptibility testing method that is as technically simple to perform as disk diffusion and produces semi-quantitative results that are measured in micrograms per milliliter (µg/ml). It is drug-specific, consists of a thin plastic antibiotic gradient strip that is applied to an inoculated agar plate, and is convenient in that it applies the principles of agar diffusion to perform semi-quantitative testing.<sup>16</sup>

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 log<sub>2</sub> dilutions by a conventional reference MIC procedure as suggested by the NCCLS. The Etest® has been compared and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations

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<sup>15</sup> The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

<sup>16</sup> Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

(see, e.g., Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer's directions for use of the Etest®**, as certain antibiotic-bacteria ("drug-bug") combinations have special testing requirements. For example, macrolides (e.g., azithromycin, erythromycin) should be tested in a normal atmosphere, not with CO<sub>2</sub>.

### ***Methods for performing antimicrobial susceptibility testing of *S. pneumoniae* with the Etest®***

The manufacturer of the Etest® indicates that when testing *S. pneumoniae*, the Mueller-Hinton agar test medium can be supplemented with either sheep or horse blood; however, it may be easier to interpret results on medium prepared with sheep blood (except when testing susceptibility to trimethoprim-sulfamethoxazole, in which case sheep blood should not be used as a supplement) [CDC, unpublished data]. This laboratory manual therefore suggests that Mueller Hinton agar with 5% sheep blood should be used when performing antimicrobial susceptibility testing of *S. pneumoniae* with the Etest® (except when testing for susceptibility to **trimethoprim-sulfamethoxazole, in which case horse blood should be used** in place of sheep blood). Either 150-mm or 100-mm plates can be used, depending on the number of Etests® used per sample (Figure 7). Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and note that although the manufacturer states that up to six Etest® strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five Etest® strips be used on a 150-mm plate.

- a) Suspend viable colonies from an overnight blood agar plate into a broth tube to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**
- b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- c) Allow the plate to dry for up to 15 minutes. **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20°C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20°C freezer.

- d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps (Figure 7.) Make sure that the printed MIC values are facing upward (*i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) **Once applied, do not move the antimicrobial gradient strips.**
- e) Incubate the plates in an inverted position in a CO<sub>2</sub>-enriched atmosphere (2%–5% CO<sub>2</sub>) for 20–24 hours at 35°C. A candle-extinction jar may be used if a CO<sub>2</sub> incubator is not available.
- Always follow the manufacturer's instructions included with each package of strips, because incubation conditions may vary by organism-antimicrobial (or “drug-bug”) combination.
- f) After incubation, there will be an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **It is important to review quality control results before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end-point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,<sup>17</sup> and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but the marks also represent increments between those standard values. The standard values (Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that if the MIC appears to be an inter-dilutional value, both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a *S. pneumoniae* isolate to penicillin, an MIC recorded from the graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml, and the organism would be interpreted as being intermediate to penicillin.

Breakpoints follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert.** NCCLS breakpoints for *S. pneumoniae*–antimicrobial combinations are included in Table 5.

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<sup>17</sup> AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abbiobdisk.com>.

## Surveillance for emerging pneumococcal resistance

Laboratories may wish to help detect the emergence of new strains of pathogens by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. This could be done, for example, on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11.

Antimicrobials of interest could include (but are not necessarily limited to): tetracycline, erythromycin, clindamycin, rifampin, ceftriaxone, amoxicillin, ciprofloxacin and vancomycin. Appropriate zone sizes can be found in NCCLS documents, which are updated regularly. Laboratorians should notify a reference laboratory of any isolates observed to have rare characteristics of non-susceptibility; for example, as of early 2002, no pneumococcus has exhibited decreased susceptibility to vancomycin [NCCLS 2002]. A list of international reference laboratories is included in Appendix 14.

## Data for decision-making

Once the laboratory has antimicrobial susceptibility patterns of *S. pneumoniae* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors. Information on pneumococcal antimicrobial resistance, together with data on the major pneumococcal serotypes responsible for disease, may become increasingly valuable to public health officials in the future, as new formulations of multivalent pneumococcal conjugate vaccines become available for global use.<sup>18</sup>

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<sup>18</sup> The Vaccine Alliance maintains information on these sorts of activities on its website: [www.vaccinealliance.org](http://www.vaccinealliance.org).

Sexually Transmitted  
Bacterial Pathogen  
for which there are  
Increasing Antimicrobial  
Resistance Concerns

*Neisseria gonorrhoeae*

## *Neisseria gonorrhoeae*

### CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

*Neisseria gonorrhoeae*, also commonly referred to as “gonococcus” or “GC”, causes an estimated 62 million cases of gonorrhea worldwide each year [Gerbase *et al.*, 1998]. Spread by sexual intercourse, *N. gonorrhoeae* may infect the mucosal surfaces of urogenital sites (cervix, urethra, rectum) and the oro- and nasopharynx (throat), causing symptomatic or asymptomatic infections. GC is always pathogenic and, if untreated, gonorrhea is a major cause of pelvic inflammatory disease (PID), tubal infertility, ectopic pregnancy, chronic pelvic pain and/or disseminated gonococcal infection (DGI). The probability of co-infection with other sexually transmitted infections (STIs) may be high in some patient populations. Neonates may acquire gonococcal infection of the conjunctiva during birth. The diagnosis of gonorrhea in older infants and young children is often associated with allegations of sexual abuse; transmission through neither nonsexual human nor fomite contact has been documented. Epidemiological studies provide strong evidence that gonococcal infections facilitate HIV transmission [Fleming and Wasserheit 1999]. Extended-spectrum cephalosporins, fluoroquinolones and spectinomycin are recognized as the most effective antibiotics for the treatment of gonorrhea in most areas of the world.

Antimicrobial resistance in *N. gonorrhoeae* is the most significant challenge to controlling gonorrhea. Gonococcal strains may be resistant to penicillins, tetracyclines, spectinomycin, and, recently, resistance to the fluoroquinolones (ciprofloxacin and ofloxacin) and the macrolide azithromycin has emerged [Handsfield 1994; Knapp *et al.* 1997; Young *et al.* 1997; CDC 1999]. Resistance to the penicillins and tetracyclines is conferred by chromosomal and/or plasmid-mediated mechanisms. Resistance to spectinomycin, fluoroquinolones and azithromycin is chromosomally mediated, and certain types of chromosomal mutations may contribute to resistance to several classes of antibiotics simultaneously.

Agents used for the treatment of bacterial infections, including co-infecting STIs, may select for resistance in *N. gonorrhoeae*. For example, whereas a 1-gram dose of azithromycin is sufficient for treatment of infections with *C. trachomatis* and *H. ducreyi*, this dose is sub-optimal for the treatment of *N. gonorrhoeae* and may result in the incidental selection and spread of resistant gonococcal strains. At the time of writing of this manual (2002), the broad-spectrum cephalosporins (ceftriaxone, cefixime, etc.) are the only class of antimicrobial agents to which gonococci have not developed confirmed resistance, although a few isolated strains have exhibited decreased susceptibility to cefixime [CDC 2000; Wang 2002].

It is of great importance to perform laboratory surveillance of antimicrobial resistance in *N. gonorrhoeae* in order to assess the effectiveness of locally recommended therapies. Only measurement of the *in vitro* susceptibilities of the infecting organism will provide objective information to help determine if a post-treatment isolate is truly resistant to the antimicrobial agent being used to treat the infection, as opposed to infection which fails to respond to treatment due to inadequate absorption of the agent, non-compliance with therapy, or re-exposure. At the population level, surveillance is key for the monitoring of local, regional and international trends in antimicrobial resistance, which can help inform and shape public health policy. Comparison between antimicrobial susceptibilities of gonococci isolated in different geographical areas provides information about the distribution and temporal spread of resistant isolates. Thus, changes in recommended antimicrobial therapies can be anticipated, and surveillance can be enhanced to guide timely changes in these therapies at the local level.

## Presumptive identification of *N. gonorrhoeae*

After the specimen has been collected from the patient, it should be labeled with a unique identifier assigned in tandem with the demographic and clinical information so it can be linked for epidemiological studies. Methods for streaking for isolation from specimen swabs, primary culture methodology, and isolate storage and transport are included in Appendices 8, 11 and 12.

Because *N. gonorrhoeae* is highly susceptible to adverse environmental conditions (as described in Table 28 of Appendix 8), strains must always be incubated at 35°–36.5°C in a humid, CO<sub>2</sub>-enriched atmosphere. Subculture colonies that appear to be gonococcal (gram-negative diplococci growing in pinkish-brown colonies 0.5 – 1 mm in diameter, see Appendix 8) from the primary selective medium to a non-selective medium, such as GC-chocolate agar with 1% defined supplement, to obtain a pure culture of the isolate. (Specimens from normally sterile sites, such as the conjunctiva, are cultured on nonselective medium for primary isolation; subculture for purity if examination of the plate shows evidence of contaminants.) If the subcultured isolate is not pure, continue to perform serial subcultures of individual colonies of gram-negative diplococci until a pure culture is obtained.

A presumptive diagnosis of *N. gonorrhoeae* originally isolated on selective medium can be made based upon colonial morphology, the observation of typical (gram-negative) diplococci in pairs, tetrads or clusters upon Gram stain or simple single stain with Loeffler's methylene blue, and a positive oxidase reaction. A presumptive diagnosis of *N. gonorrhoeae* originally isolated on nonselective medium can be made based upon these characteristics **plus** an appropriate reaction in at least one supplemental biochemical or enzymatic test (e.g., superoxol 4+ reaction, see 'Supplemental Tests'). A flowchart of tests required for presumptive identification of isolates from sites with normal flora (i.e., isolated on selective media such as MTM, ML, or GC-Lect) and isolates from normally sterile sites (i.e., isolated on nonselective medium, such as GC-chocolate agar) is presented in Figure 19.

## Oxidase test

The oxidase test uses Kovac's reagent (a 1% (wt/vol) solution of *N, N, N, N'*-tetramethyl-*p*-phenylenediamine dihydrochloride)<sup>18</sup> to detect the presence of cytochrome *c* in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. *Neisseria* species give a positive oxidase reaction, and gram-negative oxidase-positive diplococci isolated on gonococcal selective media may be identified presumptively as *N. gonorrhoeae*. Preparation of oxidase reagent and appropriate quality control methods are included in Appendix 2.

Perform an oxidase test on growth of representative colonies that stained as (gram-negative) diplococci. Because the oxidase reagent is toxic for bacteria, it is recommended to perform the oxidase test on a sterile swab and not directly on the culture plate, particularly if there are only a few suspect colonies. Alternatively, one can use filter paper in place of a swab for this test. **Do not perform the oxidase test with a Nichrome loop**, as it may produce a false-positive reaction. If a sterile swab was used to make a smear for the Gram stain (as described in Appendix 4), the swab can then be used to conduct the oxidase test. The oxidase test should only be performed on freshly grown (18–24 hour) organisms.

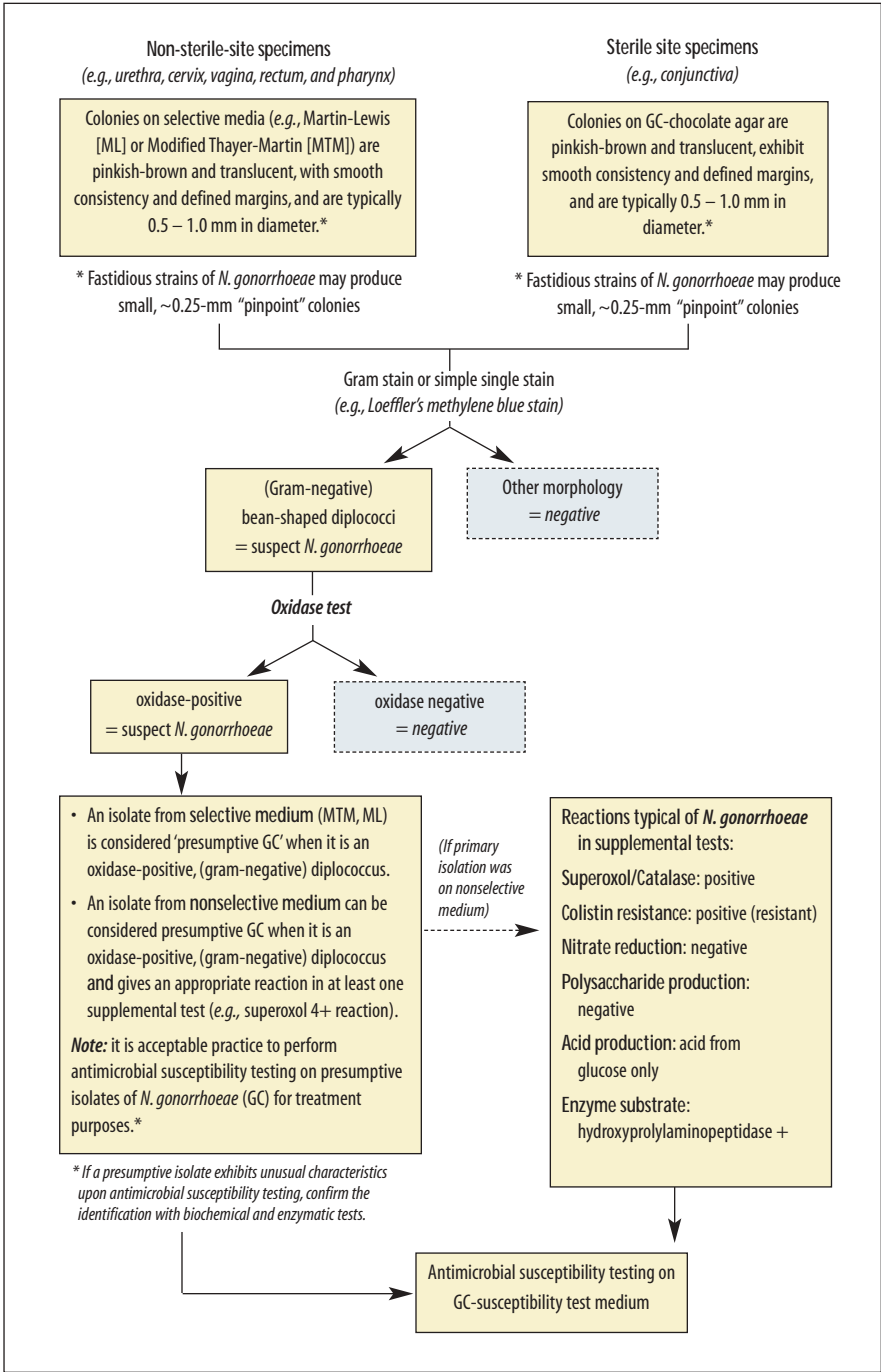
### • *Swab method for Kovac's oxidase test*

- a) Select suspect colonies from the culture plate (selective or nonselective medium) with the swab.
- b) Use a Pasteur pipette to add one drop of oxidase reagent to the swab.
- c) If the isolate is *N. gonorrhoeae*, a positive (purple) reaction should occur within 10 seconds.<sup>18</sup> (See Figure 20).

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<sup>18</sup> 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**.

FIGURE 19: Flowchart for isolation and presumptive identification of *Neisseria gonorrhoeae*



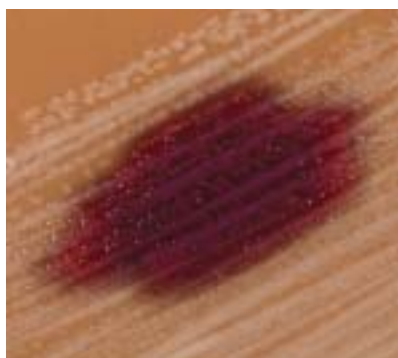
- **Moistened filter paper method for Kovac's oxidase test**

- a) Place a piece of filter paper in a petri dish.
- b) Just prior to performing the test, add one to two drops of oxidase reagent to the filter paper and allow it to absorb; the filter paper should be moist, but not wet, after the reagent has been absorbed.
- c) Using a platinum loop, a plastic loop, a sterile swab or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto the moistened filter paper. (**Do not use a Nichrome loop.**) If the isolate is *N. gonorrhoeae*, a positive (purple) reaction should occur within 10 seconds.<sup>18</sup> (See Figure 10.)

## Confirmatory identification of *N. gonorrhoeae*

If a laboratory is reporting results back to the clinical setting for treatment purposes, a presumptive diagnosis based on Gram stain and oxidase reaction is sufficient for colonies isolated on GC-selective media, and the laboratorian can continue with antimicrobial susceptibility testing of a pure culture of the isolate (presented later in this chapter). If, however, the diagnosis must be confirmed or a presumptive isolate exhibits unusual characteristics upon antimicrobial susceptibility testing (e.g. for ceftriaxone, a minimal inhibitory concentration (MIC) >0.25µg/ml, or equivalent inhibition zone diameter <35mm), the laboratorian should perform biochemical and enzymatic tests of pure culture to confirm the identification of the isolate. It is worth noting, for example, that

FIGURE 20: Kovac's oxidase test: a positive reaction on a swab



The right-hand picture shows a positive reaction on a swab that was used to harvest suspect growth and was then moistened with Kovac's oxidase reagent. The left-hand picture shows a positive oxidase direct-plate test result with Kovac's oxidase. Note that if growth is sparse, it is suggested that a laboratory **not** use the direct-plate testing method because it is toxic to gonococcal growth.

because men who have sex with men (referred to in literature as “MSM”) have higher rates of non-gonococcal neisserial infections in the urethra than do other populations, the epidemiology could lead a clinician to request a confirmed diagnosis. Another example of a situation where the diagnosis requires definitive confirmation would be a case of suspected sexual abuse; the discussion of the related social, medical and legal issues with which a laboratory could be involved goes beyond the scope of this laboratory manual.<sup>19</sup>

Figure 21 shows one pathway by which diagnosis might be confirmed with biochemical and enzymatic tests. This laboratory manual will present methods to perform tests for a reaction to superoxol reagent (or catalase reagent), colistin resistance, the production of polysaccharide from sucrose, detection of acid production with a commercial test, detection of enzyme production by a chromogenic substrate in a commercial test, and nitrate reduction. Table 6 provides a listing of reactions to a variety of tests performed on non-gonococcal species which may be mistakenly identified as *N. gonorrhoeae* based on reactions only with the acid production or enzyme substrate tests. The table includes a blank row so it may be copied and used as a sample worksheet in which to record results of confirmatory tests.

Laboratorians wishing to learn more about the concepts behind the biochemical and enzyme substrate test reactions presented here, or seeking information about other tests and methodologies in more detail, can refer to the American Society of Microbiology's *Manual of Clinical Microbiology*, or, for example, to the CDC website for clinical diagnosis of gonorrhea (<http://www.cdc.gov/ncidod/dastlr/gcdir/gono.html>).

## Biochemical and enzyme substrate supplemental tests

Species of three genera—*Neisseria*, *Kingella*, and *Moraxella* (*Branhamella*)—must be considered when examining clinical specimens or cultures for *N. gonorrhoeae*. *Neisseria* species (except *N. elongata* and *N. weaveri*) and *M. catarrhalis* are gram-negative diplococci and, in stained smears, resemble *N. gonorrhoeae*, exhibiting kidney bean- or coffee bean-shaped diplococci with adjacent sides flattened. It should be noted that is not unusual to isolate *N. meningitidis* from urethral specimens from men who have sex with men or to isolate *N. lactamica* from the throats of young children. *Kingella denitrificans* and *Moraxella* species are coccobacilli, but cells of some strains may occur as pairs and look like diplococci in smears. Thus, all of these species must be considered when identifying gram-negative diplococci in clinical specimens. Characteristics that differentiate among these genera and species are presented in Appendix 8 and Table 6. A sample listing

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<sup>19</sup> The Centers for Disease Control and Prevention (CDC) maintains a website that includes information regarding social, medical and legal issues surrounding the diagnosis of gonorrhea and with which a public health laboratory might become involved. See: <http://www.cdc.gov/ncidod/dastlr/gcdir/NeIdent/Ngon.html#Medicolegal>.

FIGURE 21: Flowchart exhibiting one algorithm for confirmatory identification of *Neisseria gonorrhoeae*

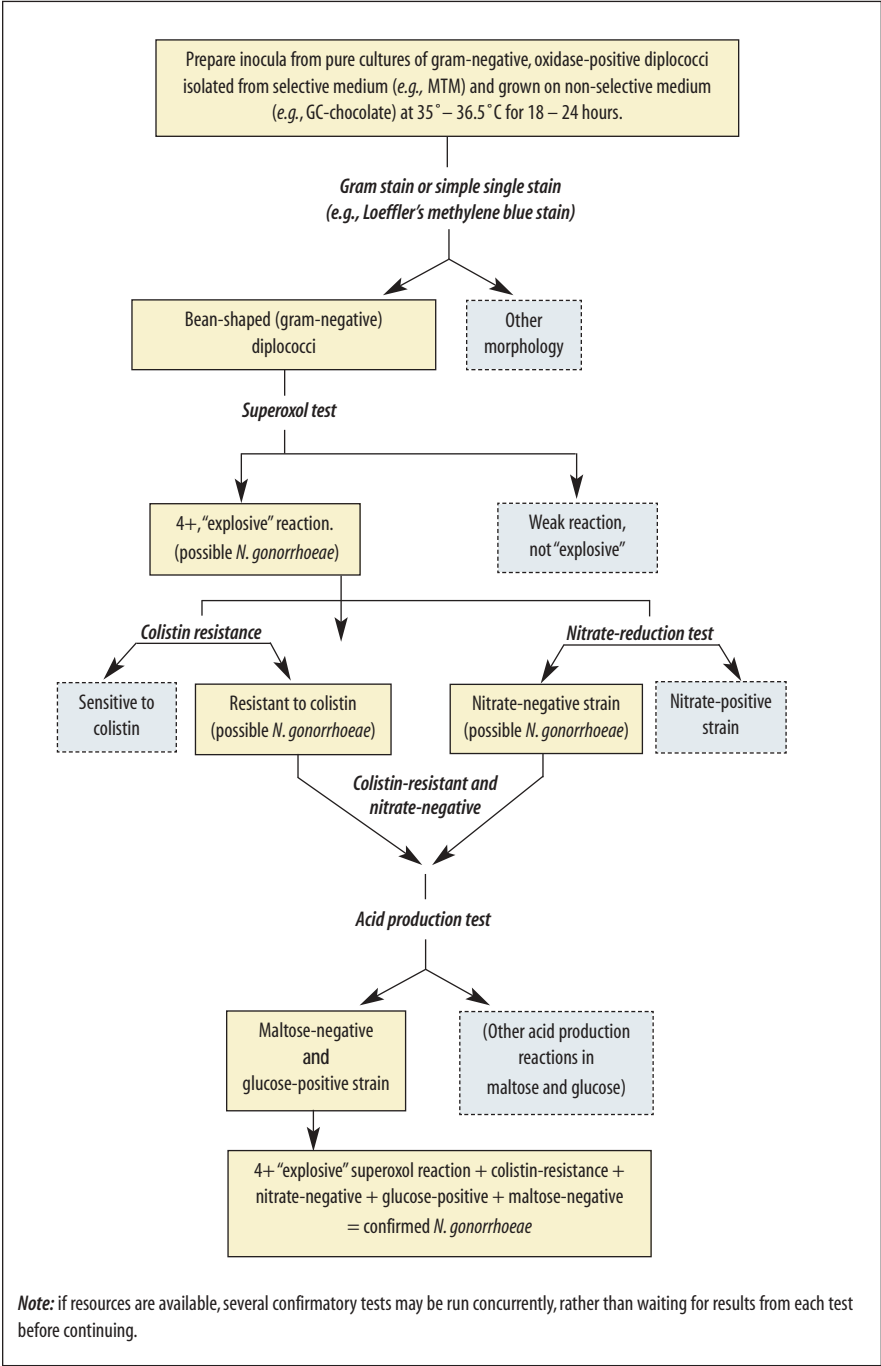


TABLE 6: Results of biochemical and enzymatic tests for *Neisseria gonorrhoeae* and related species with similar colonial morphology

Species	Cell morphology	Superoxol {catalase}	Colistin	Production of acid from:				Reduction of NO <sub>3</sub> (Nitrate)	Polysaccharide from sucrose
				GLU	MAL	LAC	SUC		
Test isolate:									
<i>N. gonorrhoeae</i> <sup>a</sup>	GND	4+ {+}	R	+	-	-	-	-	-
<i>N. meningitidis</i>	GND	1+ to 4+ {+}	R	+	+	-	-	-	-
<i>N. lactamica</i>	GND	1+ to 3+ {+}	R	+	+	+	-	-	-
<i>N. cinerea</i> <sup>b</sup>	GND	2+ {+}	(R)	-	-	-	-	-	-
<i>N. polysacharea</i>	GND	1+ to 3+ {+}	(R)	+	+	-	-	-	-
<i>N. subflava</i> <sup>b</sup>	GND	2+ {+}	(R)	+	+	-	V	-	V
<i>N. sicca</i>	GND	2+ {+}	S	+	+	-	+	-	+
<i>N. mucosa</i>	GND	2+ {+}	S	+	+	-	+	+	+
<i>N. flavescens</i>	GND	2+ {+}	S	-	-	-	-	-	+
<i>N. elongata</i>	GNR	- {-}	S	-	-	-	-	-	-
<i>M. catarrhalis</i>	GND	1+ to 4+ {+}	(R)	-	-	-	-	+	-
<i>K. denitrificans</i> <sup>c</sup>	GNC	- {-}	R	+	-	-	-	+	-

**Symbols and Abbreviations:** +, strains typically positive but genetic mutants may be negative; -, strains typically negative; V, biovar dependent (strains belonging to biovars flava and subflava do not produce acid from sucrose or produce polysaccharide from sucrose); GLU, glucose; MAL, maltose; LAC, lactose; SUC, sucrose; GND, gram-negative diplococci; GNR, gram-negative rods; GNC, gram-negative coccobacilli; R, resistant; (R), some strains resistant and may grow on gonococcal selective media; S, susceptible (insufficient data to suggest that isolates may grow on gonococcal selective media containing colistin).

<sup>a</sup> Includes *N. gonorrhoeae* subspecies kochii which exhibit characteristics of both *N. gonorrhoeae* and *N. meningitidis* (but will be identified as *N. gonorrhoeae* by tests routinely used for the identification of *Neisseria* species).

<sup>b</sup> Includes biovars subflava, flava, and perflava. Strains belonging to the biovar flava produce acid from glucose, maltose and fructose; strains belonging to the biovar subflava produce acid only from glucose and maltose.

<sup>c</sup> Coccobacillus; some strains occur in pairs which resemble gram-negative diplococci

of quality control strains for the supplemental tests described in this manual for the identification of *N. gonorrhoeae* is included in Table 7.

In a reference laboratory setting, the tests described below are best performed concurrently since they all require an inoculum prepared from fresh (18–24 hour) growth. However, when resources are limited, laboratorians may choose to screen isolates with a subset of these tests to detect isolates resembling *N. gonorrhoeae* prior to further testing. Sequential testing practices can conserve resources by limiting the use of more costly commercial tests (e.g., acid production or enzyme substrate) to only those isolates resistant to colistin and exhibiting a strong superoxol reaction. When choosing the screening approach, it is important to remember that tests performed on successive days will require a fresh (18–24 hour) subculture of the isolate.

TABLE 7: Examples of quality control (QC) strains for supplemental tests used to identify *Neisseria gonorrhoeae*

Test	Positive control	Negative control
Superoxol (or Catalase) test	<i>N. gonorrhoeae</i> ATCC 49226 [4+] <i>N. cinerea</i> ATCC 14685 [weak, 2+] (positive reaction in superoxol)	<i>K. denitrificans</i> ATCC 33394 (no reaction in superoxol)
Colistin resistance test	<i>N. gonorrhoeae</i> ATCC 49226 <i>K. denitrificans</i> ATCC 33394 (resistant to colistin)	<i>N. cinerea</i> ATCC 14685 <i>N. mucosa</i> ATCC 19696 (susceptible to colistin)
Polysaccharide production test	<i>N. polysaccharea</i> ATCC 43768 <i>N. mucosa</i> ATCC 19696 (produce polysaccharide)	<i>N. gonorrhoeae</i> ATCC 49226 <i>N. cinerea</i> ATCC 14685 (do not produce polysaccharide)
Nitrate reduction test	<i>K. denitrificans</i> ATCC 33394 <i>N. mucosa</i> ATCC 19696 (able to reduce nitrate)	<i>N. gonorrhoeae</i> ATCC 49226 <i>N. cinerea</i> ATCC 14685 (unable to reduce nitrate)
Acid production test	Use the QC strains recommended by the test manufacturer* plus <i>N. cinerea</i> . * If the manufacturer has not designated specific strains for QC: <ul style="list-style-type: none"><li>• <i>N. gonorrhoeae</i> (ATCC 49226) produces acid from glucose</li><li>• <i>N. meningitidis</i> (ATCC 13077) produces acid from glucose and maltose</li><li>• <i>N. lactamica</i> (ATCC 23970) produces acid from glucose, maltose, and lactose</li><li>• <i>N. mucosa</i> (ATCC 19696) produces acid from glucose, maltose, and sucrose</li><li>• <i>N. cinerea</i> (ATCC 14685) glucose negative, but may produce a weak glucose reaction; does not produce acid from the other sugars.</li></ul>	
Enzyme substrate test	Use the QC strains recommended by the test manufacturer.* * If the manufacturer has not designated specific strains for QC: <ul style="list-style-type: none"><li>• <i>N. gonorrhoeae</i> (ATCC 49226) produces hydroxypropylaminopeptidase.</li><li>• <i>N. meningitidis</i> (ATCC 13077) produces <math>\gamma</math>-glutamylaminopeptidase.</li><li>• <i>N. lactamica</i> (ATCC 23970) produces <math>\beta</math>-galactosidase.</li><li>• <i>M. catarrhalis</i> (ATCC 25238) produces none of these enzymes.</li></ul>	

**Note:** Laboratorians should follow QC strain designations provided by manufacturers of (commercial) tests; however, if specific strain numbers are not provided, those included in this table can be used for guidance.

## Superoxol / Catalase

The superoxol test is a simple test that uses 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) as a reagent. Reactions of superoxol with *N. gonorrhoeae* are typically “explosive” (4+, very strong), compared with weaker (2+) reactions with most non-gonococcal *Neisseria* species, and a negative reaction with *K. denitrificans*. In contrast, the catalase test is performed with 3% hydrogen peroxide and yields much weaker results. **This laboratory manual suggests performing the superoxol test (30%  $\text{H}_2\text{O}_2$ ) if the reagent is available.** This is because results with the superoxol reagent are more differential for *N. gonorrhoeae* than those obtained with the catalase reagent.

- a) Using a sterile inoculating loop or swab, remove some 18–24 hour growth from a pure culture on either selective or non-selective medium, and put it on a clean slide.<sup>20</sup>
- b) Using an eye-dropper or a pipette, place a drop of reagent onto the growth.
- c) *N. gonorrhoeae* typically has a very strong (4+), “explosive” reaction to contact with superoxol reagent, as pictured in Figure 22. Catalase will give a much weaker (1+ or 2+) reaction.
- d) Follow steps *a* and *b* to perform the superoxol/catalase test on positive and negative QC strains. (Examples of QC strains are included in Table 7.)

It should be noted that some strains of *N. meningitidis* and *M. catarrhalis* will have a strong superoxol reaction that is not ‘explosive’ upon the addition of the hydrogen peroxide but can appear as such to an eye unfamiliar with the characteristic reaction of *N. gonorrhoeae*. This test, therefore, is not definitive for *N. gonorrhoeae*, although it remains differential.

## Colistin resistance

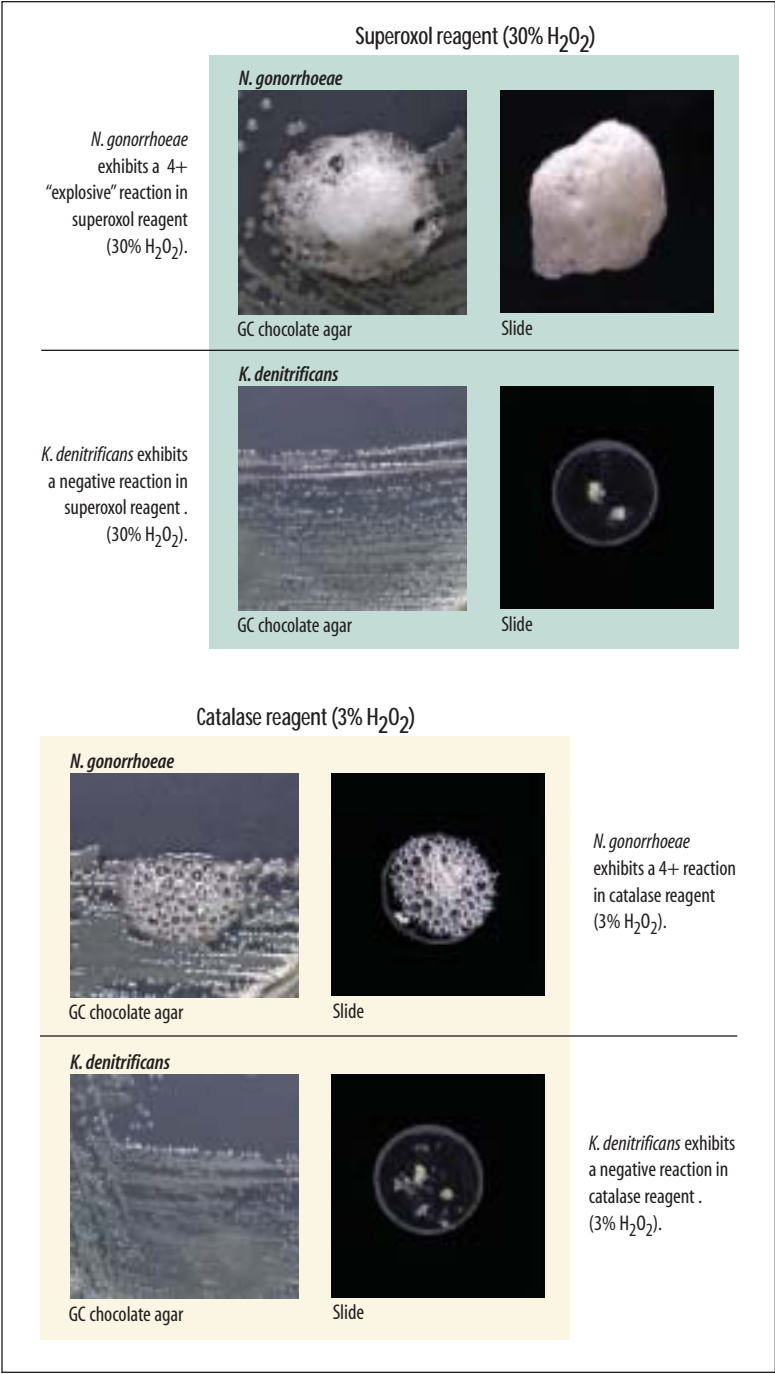
Resistance to colistin can be determined either on a selective medium containing colistin (e.g., MTM or ML), or on GC-chocolate agar using the principles of disk diffusion (with a 10 µg colistin disk). A disk diffusion method for qualitative measurement of colistin resistance is presented here.

- a) Turn a plate of medium so that it is lid-down on a table. Use a waterproof marker to divide the plate into labeled sectors for the test strain(s), the positive control and the negative control. Examples of QC strains are included in Table 7.

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<sup>20</sup>The superoxol / catalase tests can be performed directly on a plate. **However**, it should be noted that hydrogen peroxide reacts with red blood cells, although reactions have not been noted on GC-chocolate agar. If the test is to be performed on an agar plate, place a drop of the reagent on the surface of an uninoculated plate of the medium (or an area of the test plate that does not contain growth) to ensure that no reaction occurs with medium and reagent alone; if a reaction does occur, the test must be performed on a slide (or in a petri dish).

FIGURE 22: Positive and negative reactions in superoxol (30%  $\text{H}_2\text{O}_2$ ) and catalase (3%  $\text{H}_2\text{O}_2$ ) reagents



- A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the colistin resistance test at once, **and the colistin disks are from the same batch**, it is appropriate to run the positive and negative controls on only one plate.
- b) Prepare a suspension of a pure overnight culture (approximately equal to a 0.5 McFarland turbidity standard) in Mueller-Hinton broth or phosphate buffered saline (PBS).
  - c) Using a sterile swab or inoculating loop, inoculate the GC-chocolate agar plate evenly with a swab. Allow the plate to dry so that there is no visible surface moisture.
  - d) Apply a colistin disk (10 µg) to the center of the plate, tapping it down to ensure even contact with the surface. Incubate at 35°–36.5°C in 5% CO<sub>2</sub> and increased humidity for 18–24 hours.

After incubation, examine the plate for inhibition of growth around the colistin disk. *N. gonorrhoeae* is colistin-resistant, and will grow all the way up to the disk, as will all strains of *N. meningitidis*, *N. lactamica* and *K. denitrificans*. In contrast, strains of commensal *Neisseria* species, most of which are colistin-susceptible, will exhibit zones of inhibition at least 10 mm in diameter with a non-standardized inoculum. Some strains of *N. subflava* biovars, *N. cinerea*, and *M. catarrhalis* may be sufficiently resistant to colistin so as to also grow up to the disk. Thus, the colistin resistance test is not definitive for *N. gonorrhoeae* but will aid in differentiating between this species and many commensal species.

### ***Polysaccharide production test***

Some species produce a starch-like polysaccharide when grown on a medium containing sucrose. Upon addition of a drop of Gram's iodine to the growth, this starch will immediately stain dark blue-purple to brown or black. This test is easy to perform and is a useful differential test to be used in combination with others (e.g., superoxol, colistin resistance, acid production) in the identification of *N. gonorrhoeae*. **It is not possible to detect polysaccharide in the sucrose-containing medium of rapid acid-detection tests.** The methods for preparation of the medium appropriate for this test (tryptone-based soy agar [TSA] containing 1% sucrose) can be found in Appendix 2.

- a) Turn a plate of sucrose medium so that it is lid-down on a table. Use a waterproof marker to divide the plate into labeled sectors for the test strain(s), the positive control and the negative control. (Examples of QC strains are included in Table 7.)
  - A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the polysaccharide test at once, **and the**

**plates of medium are from the same batch**, it is appropriate to run the positive and negative controls on only one plate.

- b) Use a sterile swab or loop to inoculate the polysaccharide test medium with pure culture.
- Although this test is best performed on isolated colonies, because *N. gonorrhoeae* and strains of some other species do not grow well on this medium, the plate should be inoculated heavily for confluent growth so that the test can detect starch produced by pre-formed enzyme in the inoculum itself.
- c) Incubate medium at 35°–36.5°C in a CO<sub>2</sub>-enriched, humid atmosphere for 18–24 hours.
- **It is important that this test be performed on growth no more than 24 hours old.** This is because on prolonged incubation the organisms may metabolize the polysaccharide, thus resulting in a false-negative reaction.
- d) Use a Pasteur pipette, eyedropper, or inoculating loop to add one drop of Gram's iodine to growth on the plate. Isolates that produce polysaccharide will immediately turn a dark color (brown, purple, black), as shown in Figure 23.
- If the growth immediately changes color with the addition of Gram's iodine, the strain is considered "polysaccharide-positive." Examples of polysaccharide-positive organisms include *N. polysaccharea*, *N. mucosa*, *N. sicca*, and *N. flavescens*.
  - If the growth does not change color (other than acquiring the light-brown color of the iodine reagent), the reaction is negative, and the strain is considered "polysaccharide-negative." ***N. gonorrhoeae* is polysaccharide-negative**, as are, e.g., *K. denitrificans*, *M. catarrhalis*, *N. cinerea*, *N. lactamica*, and *N. meningitidis*.

Quality control should be performed with each new batch of sucrose medium or reagent. **This is particularly important because some commercial preparations of Gram's iodine will not react with the starch, yielding false-negative results.** Examples of controls for the polysaccharide-production test are listed in Table 7.

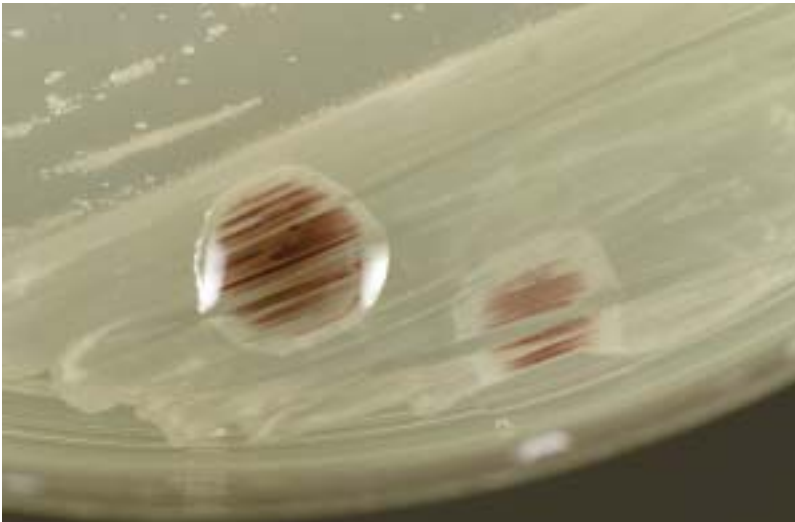
### ***Acid production test***

As of the time of writing of this laboratory manual (2002), **it is no longer advised that cystine trypticase agar (CTA) containing glucose, maltose, lactose or sucrose be used for acid production tests for *N. gonorrhoeae*.** The rationale for this shift in procedure is because many strains of *N. gonorrhoeae* produce very little acid from glucose and the color change is not observed in the CTA-sugar media, thus yielding incorrect identifications.

FIGURE 23: Positive and negative results of the polysaccharide-production test on sucrose medium

**Polysaccharide positive**

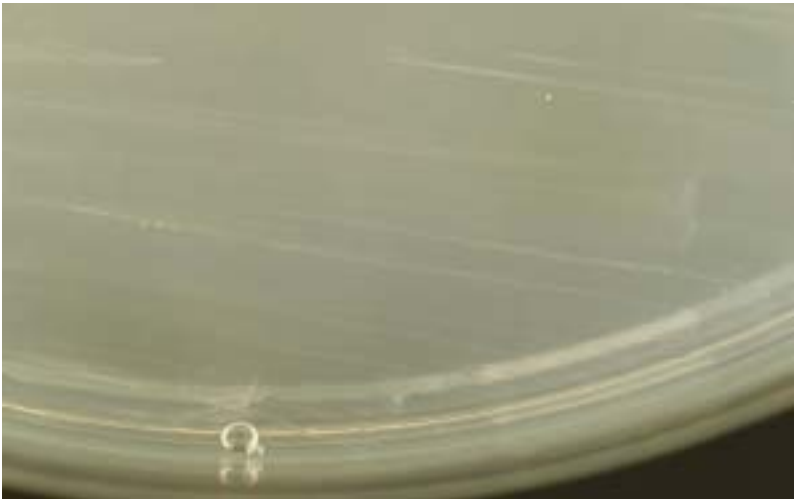
*N. polysaccharaea*



Organisms able to produce polysaccharide from sucrose turn a brown to blue-black color with the addition of Gram's iodine to growth on sucrose medium and are termed "polysaccharide-positive."

**Polysaccharide negative**

*N. gonorrhoeae*



Organisms unable to produce polysaccharide from sucrose do not undergo a color change with the addition of Gram's iodine to growth on sucrose medium and are termed "polysaccharide-negative."

(Note: polysaccharide-negative colonies may acquire the light brown-yellow color of the iodine reagent.)

Because CTA-sugar media can exhibit misleading results for some strains of *N. gonorrhoeae*, as described above, this laboratory manual advises that, if available, a commercial test be used if it is necessary to detect acid production to confirm the identification of an isolate as *N. gonorrhoeae*. Perform the test according to the manufacturer's instructions and using the manufacturer's recommendations for quality control; note that incubation of the acid production test must occur in an atmosphere **without** supplemental CO<sub>2</sub> in order to avoid false-positive results. It is important that the test chosen to detect acid production be able to differentiate between *N. gonorrhoeae* and *N. cinerea* and *M. catarrhalis*. Reaction patterns of various *Neisseria* species in the acid production test are illustrated in Figure 24.

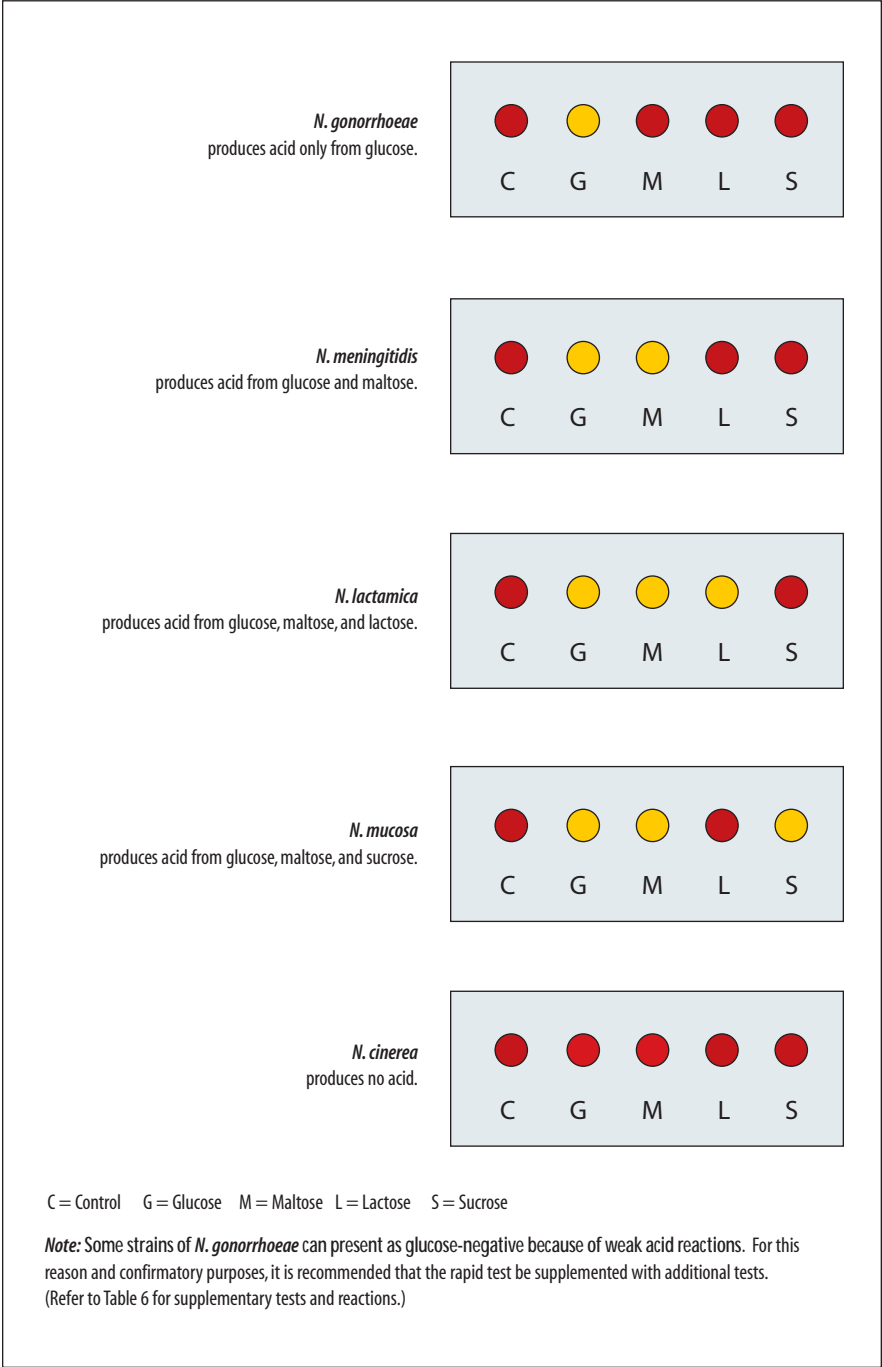
Many of the commercial acid production tests were developed to differentiate among species that routinely grow on selective media for the gonococcus, including *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *M. catarrhalis*. However, interpretive criteria provided with the product (package insert) may not provide guidance for the identification of *K. denitrificans*, *N. subflava* biovars, and *N. cinerea* strains, all of which may also grow on gonococcal selective media. The laboratory will therefore want to ensure that the product can distinguish *N. gonorrhoeae* from these other species, or perform additional tests to allow the correct identification of *N. gonorrhoeae*. Table 6 provides information to guide the choice of supplemental tests for the differentiation of *N. gonorrhoeae* from other species.

It is suggested that a strain of *N. cinerea* be included among QC strains for the acid production test (in addition to *N. gonorrhoeae* and others). Although *N. cinerea* is considered to be glucose-negative and will be listed as such in tables of acid production reactions, it does actually produce acid from glucose and then rapidly over-oxidize it to produce CO<sub>2</sub> and water; as a result, it may either appear negative or give a weak positive glucose reaction (due to residual acid produced from the glucose and not over-oxidized, and/or due to residual carbonic acid from the production of CO<sub>2</sub>), and it is therefore useful to compare this reaction to that of the *N. gonorrhoeae* control strain. In addition to *N. cinerea*, follow the quality control instructions provided by the manufacturer of the commercial kit; if the manufacturer has not provided specific QC strain designations, guidance for the selection of appropriate QC strains is provided in Table 7.

### **Enzyme substrate test**

The chromogenic enzyme substrate test detects enzymes ( $\beta$ -galactosidase,  $\gamma$ -glutamylaminopeptidase, and hydroxyprolylaminopeptidase), and is considered "chromogenic" because color changes indicate the presence or absence of certain enzymes in different *Neisseria* species. The test is commercially available and should be performed according to the manufacturer's directions. (Figure 25 in this laboratory manual shows the Gonocheck-II®.) Because most enzyme substrate tests were developed to differentiate only among the organisms believed to grow on media selective for *N. gonorrhoeae*, documentation provided with the product is

FIGURE 24: Acid production commercial test kit results for *Neisseria gonorrhoeae* and related organisms



usually limited to distinguishing between *N. gonorrhoeae* (which produces only hydroxyprolylaminopeptidase), *N. meningitidis* (which produces  $\gamma$ -glutamyl-aminopeptidase), *N. lactamica* (which produces  $\beta$ -galactosidase), and *M. catarrhalis* (which produces none of these three enzymes). It is now known that strains of several commensal *Neisseria* species can grow on selective GC media and also produce only hydroxyprolylaminopeptidase. The chromogenic enzyme substrate test is therefore not definitive for the identification of *N. gonorrhoeae*. Table 6 provides information to guide the choice of supplemental tests for the differentiation of *N. gonorrhoeae* from other species. Follow the quality control instructions provided by the manufacturer of the commercial kit; if the manufacturer has not provided specific QC strain designations, guidance for the selection of appropriate QC strains is provided in Table 7.

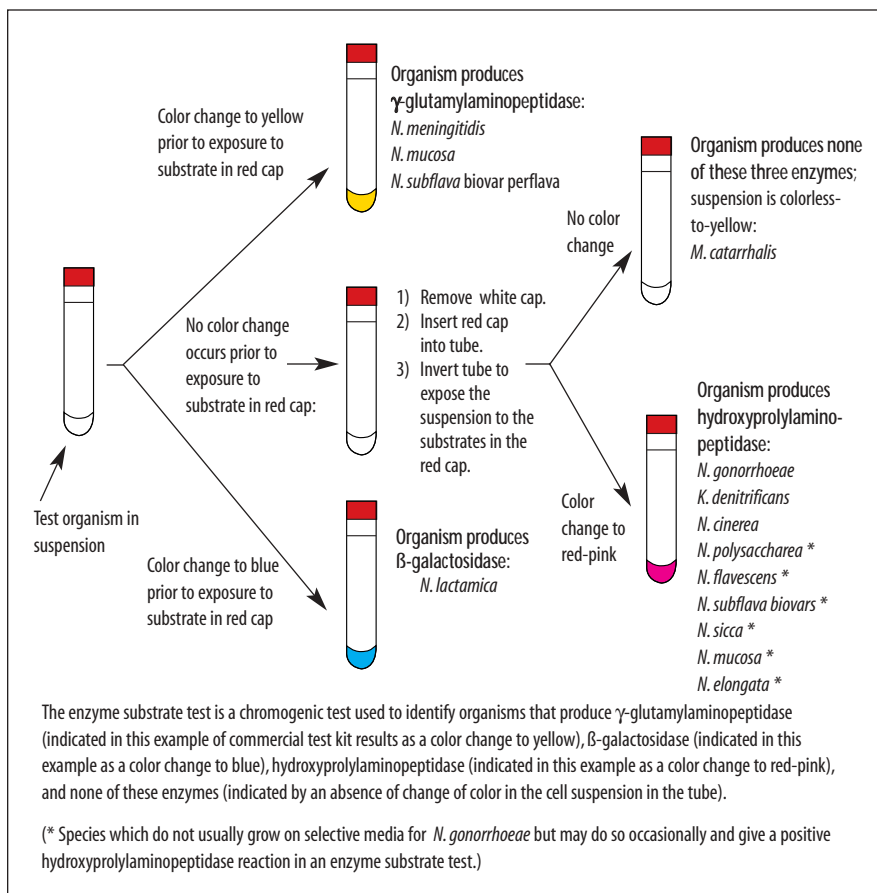
### **Nitrate reduction test**

The nitrate reduction test is available commercially or can be made easily in the laboratory. This test distinguishes between species that can reduce nitrate ( $\text{NO}_3$ ) to nitrite ( $\text{NO}_2$ ) or nitrogenous gases. In the context of this chapter, the test is useful for differentiating between strains of *N. gonorrhoeae* (nitrate-negative) and *K. denitrificans* or *M. catarrhalis* (two nitrate-positive species sometimes misidentified as *N. gonorrhoeae*).

The nitrate reduction test uses a medium containing nitrate and three different reagents: sulfanilic acid ("Nitrate Reagent A"),  $\alpha$ -naphthylamine ("Nitrate Reagent B"), and zinc powder ("Zn<sup>+2</sup> dust"). Bacteria able to reduce nitrate from the medium into either nitrite or into nitrogenous gases are "nitrate-positive," while bacteria that lack enzymes to reduce nitrate are "nitrate-negative."

In practical terms, the nitrate reduction test centers around the colorimetric detection of nitrite in the test medium. Nitrite forms a compound with sulfanilic acid, which when reacted with  $\alpha$ -naphthylamine gives a pink-to-red color depending upon the concentration of nitrite in the medium; the addition of Nitrate Reagents A and B is therefore only able to detect the presence of nitrite in the medium. If a pink-red color is detected after the addition of Nitrate Reagents A and B, the organism is considered to be "nitrate-positive." However, if there is no color change in the medium after the addition of these reagents, it is necessary to determine whether nitrate was ever reduced to nitrite, or whether the nitrite produced was completely reduced to nitrogenous gases. This is accomplished by using a small amount of zinc powder, which chemically catalyzes the reduction of nitrate to nitrite and nitrite to nitrogenous gases. (It is therefore critical to use only a very small amount of zinc powder so that if nitrate has not been reduced by enzymes produced by the bacteria, the reaction catalyzed by the zinc powder is not so strong as to reduce the nitrate completely to nitrogenous gases so rapidly that it is not possible to detect the nitrite produced in the catalytic reaction in the medium.) **Nitrate-negative strains will exhibit a color change to red after**

FIGURE 25: Reactions of *Neisseria gonorrhoeae* and related organisms in a commercial enzyme substrate test



**incubation with zinc powder** (nitrate is reduced to nitrite by the zinc powder, and the nitrite is detected by Nitrate Reagents A and B already in the medium, yielding a color change to pink-red). Nitrate-positive strains do not exhibit a color change after incubation with zinc powder because nitrate in the medium will have already been reduced beyond nitrite to nitrogenous gases. To summarize:

- Bacteria that reduce nitrate to nitrite may be identified when addition of Nitrate Reagents A and B causes the medium to change color from clear to pink-red; no additional testing with zinc powder is required. Results should be recorded as “nitrate-positive.”
- Bacteria that reduce nitrate to nitrite and then further reduce the nitrite to nitrogenous gases are identified when there is no color change in the medium after either the addition of Nitrate Reagents A and B, or after incubation with zinc powder. Results should be recorded as “nitrate-positive.”

- Bacteria unable to reduce nitrate at all are identified when there is no color change with the addition of Nitrate Reagents A and B, but there **is** a color change in the medium from clear to pink-red after incubation with zinc powder. Results should be recorded as “nitrate-negative.”

The nitrate test is performed in a standard nitrate broth which is inoculated heavily to give a dense suspension of organisms because many *Neisseria* species may not grow in this medium; the reaction for these species will therefore depend upon preformed enzymes in the inoculum. The test must be performed exactly as described; if not performed correctly, the test results may be inaccurate and an incorrect identification made. A schematic representation of the nitrate reduction test is shown in Figure 26. Media and reagents required for this test are described in Appendix 2.

Nitrate reduction occurs only under anaerobic conditions; it is therefore important to ensure a low surface-area to depth ratio to limit the diffusion of oxygen into the medium during the test. These conditions will be met by dispensing 5 ml of medium into a 13 mm diameter screw-cap tube.

It is important to run a medium control and both negative- and positive- controls as the test is complex and the controls have known outcomes to indicate if the media and reagents are reacting appropriately. **Quality control tests should be performed each time clinical isolates are tested**, using QC strains included in Table 7.

### Methods

- a) Using colonies from a fresh, pure culture on GC-chocolate agar, prepare a heavy suspension in nitrate broth.
- b) Remove the screw-cap top from the tube of nitrate test medium and inoculate the medium to give heavy turbidity. Replace the screw-cap top.
- c) Incubate the inoculated tubes and an uninoculated medium control tube at 35°–36.5°C (without supplemental CO<sub>2</sub>) for 48 hours.
- d) After incubation for 48 hours, remove the screw-cap top from the tube. Add 5 drops of Nitrate Reagent A to each tube (including the uninoculated control medium). Shake each tube gently back and forth to mix Reagent A with the medium, add 5 drops of Nitrate Reagent B to each tube (again including the uninoculated control medium), and again shake each tube gently back and forth to mix Reagent B with the medium.
  - **If the uninoculated control medium turns pink-red**, the test is invalid, and a new batch of media must be prepared.
  - **If the uninoculated control medium shows no color change**, proceed to step e.

- e) Examine the test medium and controls for a pink-red color; this color should develop within a few minutes if the medium is still warm. The reaction may take a little longer if the medium has cooled before the reagents are added.
- The negative control medium should show no color change.
  - The positive control medium may or may not exhibit a color change to pink-red, depending upon whether nitrate was reduced to nitrite or further reduced to nitrogenous gases.
  - **If the test medium turns pink-red after the addition of Nitrate Reagents A and B**, the reaction is positive and the test is completed. If a pink-red color develops, do not perform step *f* and **record the reaction as nitrate-positive**.
- f) If the medium is still colorless after the addition of Nitrate Reagents A and B, add a very small amount of zinc powder to the medium. (A convenient method to estimate the amount of zinc powder required for the test is to use the sharp point of a knife to pick up the powder; the pile of zinc powder should not exceed 4–5 mg, or 2–3 mm in diameter.) Shake the tube vigorously back and forth to mix well, and allow it to stand at room temperature for 10–15 minutes.
- If the negative control turns pink-red after the addition of zinc powder, the amount of zinc added is sufficient for the reaction to occur (and not so much as to cause rapid over-reduction of nitrate to nitrogenous gases). Continue by interpreting the reactions in the test media.
  - **If the medium remains colorless after the addition of zinc powder**, the test result is positive (nitrate has been reduced to nitrite and further reduced to nitrogenous gases). Record the result for the isolate as “nitrate-positive.”
  - **If the medium turns pink-red after the addition of zinc powder**, the result is negative. Record the result for the isolate as “nitrate-negative.”  
*N. gonorrhoeae* is nitrate-negative.

No identification of genus or species can be made on the basis of any of the above biochemical and enzymatic tests alone, but performing a combination (*e.g.*, as presented in Figure 21) can lead to a definitive identification of *N. gonorrhoeae*.

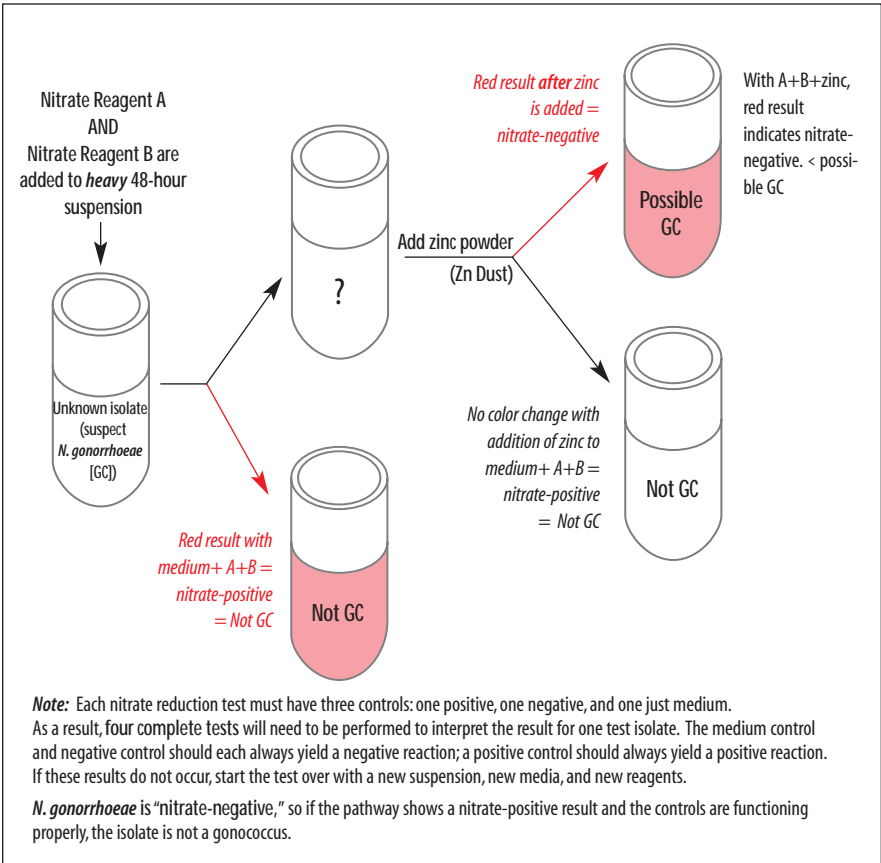
## Antimicrobial susceptibility testing of *N. gonorrhoeae*

The methods presented in this laboratory manual are those recommended by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),<sup>21</sup> although a variety of methods are used internationally to determine antimicrobial susceptibilities of *N. gonorrhoeae*.

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<sup>21</sup> Formerly known as the “National Committee for Clinical Laboratory Standards,” NCCLS is now known solely by its acronym.

FIGURE 26: Schematic representation of the nitrate reduction test



These methodologies are currently (2002) being reviewed by the International Collaboration on Gonococci (ICG), and it is possible that some modifications will be made to the methods described in this document.<sup>22</sup>

Minimal inhibitory concentration (MIC) determination by the agar dilution method is the reference method ("gold standard") for determining the antimicrobial susceptibilities of *N. gonorrhoeae* isolates. However, this method is complex to perform, and so is beyond the scope of this manual.<sup>23</sup> Antimicrobial susceptibilities can also be determined by the disk diffusion test, or MICs can be obtained with the Etest® (AB Biodisk). This document presents the methods for antimicrobial susceptibility testing of *N. gonorrhoeae* with the antimicrobial agents

<sup>22</sup> International gonorrhea reference laboratories can provide additional information on ICG activities; contact information for these laboratories is included in Appendix 14.

<sup>23</sup> Laboratorians interested in learning more about agar dilution antimicrobial susceptibility test methods may contact an ICG reference laboratory (Appendix 14).

currently recommended by WHO for the primary therapy of gonorrhea: ciprofloxacin, azithromycin, ceftriaxone, cefixime, and spectinomycin [WHO 2001].

Factors such as testing medium, inoculum size, incubation atmosphere, and antimicrobial disk concentrations may affect the antimicrobial susceptibility values obtained. Thus, quality control is of great importance and, with every test run, laboratory personnel must include reference strains with known antimicrobial susceptibilities to ensure that the susceptibility results for test isolates are accurate. It should be noted that for methods that determine MICs, the MIC will be accurate plus or minus ( $\pm$ ) one dilution. For example, an organism with an MIC of penicillin of 0.25  $\mu\text{g/ml}$  may exhibit an MIC of 0.125  $\mu\text{g/ml}$  to 0.5  $\mu\text{g/ml}$ , but it would be found upon repeated testing that most antimicrobial susceptibility values (*i.e.*, the modal MIC) for this organism and drug would be 0.25  $\mu\text{g/ml}$ . Disk diffusion results (*i.e.*, inhibition zone diameters, mm) exhibit a similar normal distribution upon repeated testing of the same isolates. It is important to keep these variations of measurement in mind as laboratories typically perform only one complete set of antimicrobial susceptibility tests per isolate, and not repeated measures for the same antimicrobial agent unless there is a specific reason to do so, such as confirming an unusual antimicrobial susceptibility result.

WHO has recommended a number of isolates for quality control (QC), although these do not adequately represent the variety of resistance patterns now known to exist for *N. gonorrhoeae*. Consequently, most international laboratories have included additional QC strains exhibiting resistance and intermediate resistance to fluoroquinolones and emerging resistance to azithromycin. Only one strain, *N. gonorrhoeae* ATCC 49226, is designated by NCCLS for QC of antimicrobial susceptibility testing of gonococcal isolates. At the Centers for Disease Control and Prevention (CDC), the NCCLS-recommended QC strain and supplemental QC strains are routinely made available to investigators (see Appendix 14 for contact information). Strains of *N. gonorrhoeae* are currently being tested under ICG sponsorship to establish an international reference panel for QC of antimicrobial susceptibility testing that represents the known range of resistances in this species.

Once the susceptibility of a gonococcal strain to an antimicrobial agent has been measured *in vitro*, the strain is then classified as susceptible, intermediate, or resistant to each antimicrobial agent tested to indicate whether the infection may either respond fail to respond to therapy with that agent. For clinical applications (*i.e.*, prescribing appropriate therapy for individual patients), antimicrobial susceptibilities are always interpreted strictly according to standardized guidelines, such as the NCCLS interpretive criteria. These criteria must be specific for the dose of the agent used to treat the infection [Knapp *et al.* 1995]. For example, NCCLS criteria for the interpretation of susceptibility of *N. gonorrhoeae* to ciprofloxacin were developed to correspond to treatment with the recommended 500-mg of ciprofloxacin in a single oral dose; assessment of treatment efficacy of a single oral dose of 250-mg of ciprofloxacin would require the development of separate interpretive criteria.

Organism-antimicrobial-dose interactions are categorized into one of two levels of classification based on the clinical efficacy of the antimicrobial agent. One level applies to antimicrobial agents to which an organism has not yet developed clinically significant resistance, and uses the categories “susceptible” and “decreased susceptibility.” The second level is used when the organism has developed clinically significant resistance resulting in failure of the infection to respond to therapy with the recommended dose of the antimicrobial agent (“treatment failures”), and uses the categories “susceptible”, “intermediate,” and “resistant.” For example:

- At the time of writing (2002), gonococcal infections have not been confirmed to fail to respond to therapy with extended-spectrum cephalosporins, such as cefixime (400-mg in a single oral dose). The NCCLS has established an interpretive criterion of “susceptible” as an MIC of  $\leq 0.25$  mg/ml of cefixime (corresponding disk diffusion zone of inhibition diameter with a 5- $\mu$ g cefixime disk,  $\geq 31$  mm). Organisms with a higher MIC (or smaller inhibition zone diameter) are classified as exhibiting “decreased susceptibility” to cefixime.
- When infections fail to respond to recommended therapies with specific antimicrobial agents, a “resistant” category is established for that organism-antimicrobial-dose combination by NCCLS. Breakpoints are set for *in vitro* determination of this category based on testing of a variety of isolates resistant to the recommended therapeutic treatment. For example, gonococcal infections caused by organisms for which the ciprofloxacin MICs are  $\geq 1.0$  mg/ml (corresponding disk diffusion zone diameter of inhibition with a 5 mg ciprofloxacin disk,  $\leq 27$  mm) have failed to respond to therapy with the WHO-recommended single oral ciprofloxacin dose of 500-mg. NCCLS had previously defined the “susceptible” breakpoint for ciprofloxacin as an MIC of  $\leq 0.06$  mg/ml (zone inhibition diameter  $\geq 41$  mm), so the “intermediate” designation applies to those isolates for which the MICs are in the range between the susceptible and resistant categories, *i.e.*,  $0.125 \mu\text{g/ml} - 0.5 \mu\text{g/ml}$  (28 mm – 40 mm). For gonococcal infections, it should be noted that organisms in the “intermediate” category for an antimicrobial agent have rarely been associated with confirmed instances of treatment failure with that agent.

NCCLS interpretive criteria are designed to define antimicrobial susceptibility test result categories when NCCLS methodology is used to perform the tests, as presented in this laboratory manual.<sup>24</sup> The additional QC reference strains included in this laboratory manual for antimicrobial agents not currently included in NCCLS guidelines have been validated by the Gonorrhea Research Branch (*Neisseria* Reference Laboratory) at the CDC, and may be used alongside NCCLS criteria with the methods presented here until an ICG-sponsored international QC

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<sup>24</sup> NCCLS methods are presented in this document, and are strongly recommended. However, if a laboratory uses different antimicrobial susceptibility testing methodologies for *N. gonorrhoeae*, and all quality control references are consistently in check with the NCCLS interpretive criteria for QC strain ATCC 49226, the laboratory may consider interpreting results for the alternate testing methodology according to the NCCLS interpretive criteria.

reference panel is designated. Tables 9 and 10 provide summaries of QC ranges and interpretive criteria for clinical isolates.<sup>25</sup>

In resource-limited geographic areas or in local clinical laboratories, **antimicrobial susceptibility test results should be determined for current antimicrobial therapies and also the alternate antimicrobial agent(s) that would be used if resistance emerged to the current regimen.** Not all local laboratories will have the capacity to perform antimicrobial susceptibility testing on isolates. National or large regional laboratories acting in the capacity of a reference laboratory should be able not only to provide assistance to local laboratories and health authorities (clinical applications), but also to perform the most extensive susceptibility testing to a broad range of antimicrobial agents in order to compare susceptibilities of isolates at the regional, national and international levels (surveillance activities).<sup>26</sup> In a local laboratory, if it is not feasible to perform prospective surveillance, the laboratory should at least determine susceptibilities of post-treatment “treatment failure” isolates which could either be truly resistant treatment failures or else susceptible isolates acquired by re-infection. If a laboratory is unable to perform antimicrobial susceptibility testing, isolates should be sent to a laboratory that can perform such testing. (Methods for preservation and storage of isolates are included in Appendix 11; transport of isolates is addressed in Appendix 12.)

In addition to providing immediate assistance to local and regional laboratories and public health authorities in efforts to control gonorrhea by determining antimicrobial susceptibilities to the recommended therapies, reference laboratories may want to conduct more extensive antimicrobial susceptibility testing in order to develop a global perspective on antimicrobial resistance in *N. gonorrhoeae*. Determination of antimicrobial susceptibilities to a wide range of agents—penicillin, tetracycline, spectinomycin, extended-spectrum cephalosporins (e.g., ceftriaxone and cefixime), fluoroquinolones (e.g., ciprofloxacin, ofloxacin, and levofloxacin), and the macrolide azithromycin—allows for the comparison of isolates from the population served by the testing laboratory with isolates from other regions.

Laboratory-based surveillance for antimicrobial resistance may be conducted at one of two basic levels. When resources are limited, surveillance may be performed for susceptibilities to antimicrobial agents being used for primary and secondary therapy of gonorrhea, i.e., the primary agent being used to treat infections and the alternative therapeutic agent(s) that would be used to treat infections not treated effectively by the primary regimen. In this instance, antimicrobial susceptibilities

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<sup>25</sup> If antimicrobial susceptibility test QC results for a locally developed testing method are consistent but do not agree with those obtained by NCCLS-recommended methods, the testing laboratory may want to consult with the ICG for assistance with the development of standard interpretive criteria appropriate to the situation.

<sup>26</sup> Laboratorians interested in learning more about the methods used for the surveillance of antimicrobial resistance in *N. gonorrhoeae* isolates can find links to various protocols through the following internet address: <http://www.cdc.gov/ncidod/dastlr/gcdir/gono.html>

would be interpreted by the standards used for clinical applications, *e.g.*, by NCCLS standards.

When investigators wish to compare the antimicrobial susceptibilities of *N. gonorrhoeae* strains in their geographic locality with those in other geographic areas, susceptibilities are usually determined to a larger number of antimicrobial agents than those used locally for treatment. A typical panel might include the following: penicillin, tetracycline, spectinomycin, an extended-spectrum cephalosporin (*e.g.*, ceftriaxone or cefixime), a fluoroquinolone (*e.g.*, ciprofloxacin, ofloxacin, or levofloxacin), and a macrolide (*e.g.*, azithromycin). For broad surveillance purposes, gonococcal isolates are described first by their susceptibilities to penicillin and tetracycline (although these drugs should not be used to treat gonorrhea) and by a simple test to detect the production of  $\beta$ -lactamase (described below). This is because, based on the level of resistance to penicillin and tetracycline and the detection of  $\beta$ -lactamase, it is possible to predict whether the mechanisms of resistance to penicillin and tetracycline are chromosomally mediated or plasmid-mediated.

A specialized classification and terminology with standard acronyms has been developed to describe patterns of penicillin-tetracycline resistance and designate penicillin-tetracycline resistance phenotypes, as presented in Table 8. Organisms that are  $\beta$ -lactamase-negative and resistant to penicillin but not tetracycline, for example, use the NCCLS designation “penicillin-resistant,” and are designated “PenR.” Other acronyms do not use NCCLS designations in their names, although NCCLS methods are used to identify the resistances. For example, “CMRNG” (chromosomally mediated resistant *N. gonorrhoeae*) describes organisms that have chromosomally mediated resistance to **both** penicillin (MIC  $\geq$  2.0 mg/ml, or equivalent inhibition zone diameter  $\leq$  26 mm) **and** tetracycline (MIC  $\geq$  2.0 mg/ml, or equivalent inhibition zone diameter  $\leq$  30 mm) **and** do not produce  $\beta$ -lactamase. It should be noted that while plasmid-mediated resistance to penicillin can be detected and confirmed with a simple test to detect  $\beta$ -lactamase, plasmid-mediated resistance to tetracycline can only be identified presumptively with susceptibility results and must be confirmed with a complex test demonstrating the presence of the TetM-conjugative plasmid (*e.g.*, by laboratories performing molecular epidemiologic testing).

The basic penicillin-tetracycline resistance phenotype acronym characterizes susceptibilities only of penicillin and tetracycline. For other therapeutic agents, NCCLS (or equivalent) standardized criteria are used to classify susceptibilities to these agents, and antimicrobial resistance (including “intermediate” or “decreased susceptibility” categories) to these additional antimicrobial agents is appended to the penicillin-tetracycline resistance phenotype. For example, a CMRNG isolate exhibiting resistance to ciprofloxacin (CipR) would be cited as “CMRNG, CipR.” Such descriptive designations permit one to rapidly appreciate the fact that ciprofloxacin resistance is occurring in an organism already resistant to penicillin

TABLE 8: Phenotypic designations of *Neisseria gonorrhoeae*

Phenotype	Phenotype definition	$\beta$ -lactamase results and specific MIC values associated with phenotype definitions for <i>Neisseria gonorrhoeae</i> <sup>a, b</sup>
Susceptible	Isolates exhibiting <i>either</i> susceptibility <i>or</i> intermediate resistance to <i>both</i> penicillin and tetracycline	$\beta$ -lactamase negative isolate exhibiting: <ul style="list-style-type: none"> <li>• Susceptibility to penicillin [MIC &lt; 2.0 <math>\mu</math>g/ml (&gt;26 mm)]</li> <li>• Susceptibility to tetracycline [MIC &lt; 2.0 <math>\mu</math>g/ml (&gt;30 mm)]</li> </ul>
PPNG	Penicillinase-producing <i>Neisseria gonorrhoeae</i>	$\beta$ -lactamase positive isolate. Approximately six $\beta$ -lactamase plasmids have been identified in <i>N. gonorrhoeae</i> , most commonly: <ul style="list-style-type: none"> <li>• “Asian” = 4.4 megadaltons (Mda) (7.2 kb)</li> <li>• “African” = 3.2 Mda (5.3 kb)</li> <li>• “Toronto” = 3.05 Mda (4.7 kb)</li> </ul> (PPNG is defined only by production of $\beta$ -lactamase and not by MICs of penicillin.) <sup>c</sup>
TRNG	Tetracycline resistant <i>Neisseria gonorrhoeae</i>	$\beta$ -lactamase negative isolates possessing a TetM-containing conjugative plasmid. TRNG isolates will exhibit both: <ul style="list-style-type: none"> <li>• Susceptibility to penicillin [MIC &lt; 2.0 <math>\mu</math>g/ml (&gt;26 mm)]</li> <li>• Resistance to tetracycline with MIC <math>\geq</math> 16.0 <math>\mu</math>g/ml (<math>\leq</math>20 mm)</li> </ul> Presumptive identification of this phenotype is based on MICs of penicillin and tetracycline. Confirmatory identification of TRNG (TetM and subtyping) is by PCR
PP/TR	Penicillinase-producing, tetracycline resistant <i>Neisseria gonorrhoeae</i>	$\beta$ -lactamase positive isolates of <i>N. gonorrhoeae</i> exhibiting: <ul style="list-style-type: none"> <li>• Resistance to tetracycline with MIC <math>\geq</math> 16.0 <math>\mu</math>g/ml (&lt;20 mm)</li> </ul>
PenR	Chromosomally mediated resistance to penicillin	$\beta$ -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> <li>• Resistance to penicillin with MIC <math>\geq</math> 2.0 <math>\mu</math>g/ml (<math>\leq</math>26 mm)</li> <li>• Susceptibility to tetracycline [MIC &lt; 2.0 <math>\mu</math>g/ml (&gt;30 mm)]</li> </ul>
TetR	Chromosomally mediated resistance to tetracycline	$\beta$ -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> <li>• Susceptibility to penicillin [MIC &lt; 2.0 <math>\mu</math>g/ml (&gt;26 mm)]</li> <li>• Resistance to tetracycline with an MIC range of 2.0 <math>\mu</math>g/ml - 8.0 <math>\mu</math>g/ml (20 – 30mm)</li> </ul>
CMRNG	Chromosomally mediated resistant <i>Neisseria gonorrhoeae</i>	$\beta$ -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> <li>• Resistance to penicillin with MIC <math>\geq</math> 2.0 <math>\mu</math>g/ml (<math>\leq</math>26 mm)</li> <li>• Resistance to tetracycline with MIC <math>\geq</math> 2.0 <math>\mu</math>g/ml (<math>\leq</math>30 mm)</li> </ul>

<sup>a</sup> **Note:** Some TRNG may exhibit tetracycline MICs <16.0  $\mu$ g/ml, and some TetR isolates may exhibit tetracycline MICs  $\geq$ 16.0  $\mu$ g/ml. The difference between TRNG and TetR can only be confirmed by a test to determine the presence or absence of the TetM plasmid.

<sup>b</sup> **Note:** For some research purposes, a breakpoint of 1.0  $\mu$ g/ml of penicillin and tetracycline is used to differentiate more equitably between (penicillin and tetracycline) susceptible isolates and isolates belonging to the CMRNG group of organisms [Rice and Knapp 1994].

<sup>c</sup> **Note:** For PPNG isolates, MICs for penicillin are typically high ( $\geq$ 8.0  $\mu$ g/ml) ( $\leq$ 20 mm); however, it is possible for them to be lower and have larger zones of inhibition as well. Some PPNG isolates have MICs as low as 0.25  $\mu$ g/ml of penicillin but are still  $\beta$ -lactamase positive.

and tetracycline. The use of penicillin-tetracycline resistance phenotypes also has practical applications for monitoring susceptibilities to the extended-spectrum cephalosporins: gonococcal isolates exhibiting chromosomally mediated, high levels of resistance to penicillin (PenR) or penicillin and tetracycline (CMRNG) exhibit higher—but still susceptible—MICs of ceftriaxone and cefixime.

Aggregation and analysis of phenotypic data permit investigators to monitor changes in the prevalence of resistant strain populations and their geographic patterns of spread, and these surveillance tools may be used to help anticipate the need to revise treatment recommendations before resistance becomes endemic in a region and undermines the effectiveness of local gonorrhea control measures.

### ***Further characterization of resistant strains***

An area of research in which reference laboratories may be interested in participating is the further subtype characterization<sup>27</sup> of isolates exhibiting the same antimicrobial resistance phenotypes. Subtyping methods are resource-intensive, however, and so it is not expected that every reference laboratory will be able to adopt these techniques. Genotypic and phenotypic subtyping characterizes individual strains and facilitates a refined interpretation of the antimicrobial resistance data. By assigning strain subtype designations, investigators may be able to differentiate between the strain types which are sporadically imported and coincidentally exhibit the same resistance phenotype as a local strain. Strain subtyping coupled with information about social-sexual networks may facilitate proactive disease control interventions.

### **Methods for detecting antimicrobial resistance in *N. gonorrhoeae***

As detailed above, there are two different approaches taken when defining for what antimicrobial agents susceptibility tests should be performed. When performing antimicrobial susceptibility testing for clinical purposes, susceptibilities should be determined to the antimicrobial agents currently used for treatment of gonorrhea and the alternate antimicrobial agent(s) that would be prescribed if the primary course were to be ineffective. When performing antimicrobial susceptibility testing for surveillance purposes, however, the clinical testing is supplemented with an expanded panel of antimicrobial agents in conjunction with  $\beta$ -lactamase testing, providing the laboratory with phenotypic data appropriate for international comparisons.

Tests identifying gonococcal strains that produce  $\beta$ -lactamase are used in conjunction with MICs as an integral component of surveillance to differentiate between chromosomally mediated and plasmid-mediated resistance to penicillin for

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<sup>27</sup> Examples of phenotypic typing include auxotyping (determination of nutrients required for growth of a strain), serotyping,  $\beta$ -lactamase plasmid typing, and TetM plasmid typing. Examples of genotypic typing include Lip subtyping, RFLP-related typing, and Opa typing.

*N. gonorrhoeae*, as explained above. The nitrocefin test is a qualitative test used to detect production of  $\beta$ -lactamase; it can be performed using the same culture on GC-chocolate agar used to prepare the inoculum for MIC (or disk diffusion) tests.

### ***Test for $\beta$ -lactamase production by *N. gonorrhoeae****

The most reliable way to detect  $\beta$ -lactamase-producing strains of *N. gonorrhoeae* is to use the nitrocefin test. Reactions are strongest when the test is performed on cultures recently removed from an incubator and still warm. The nitrocefin test is performed either with a liquid reagent or with a treated disk. Because the liquid reagent can be expensive, the disk method is preferable if relatively few isolates are to be tested. Positive and negative controls should be run each time this test is performed. Positive and negative control strains may be selected from among those listed in Table 7.

#### ***Nitrocefin disk method***

- a) Use sterile forceps or tweezers to place a nitrocefin disk on a clean slide.
- b) Add a drop of distilled water to the disk and allow it to absorb so the disk is moistened, but not wet.
- c) Touch a sterile swab or loop to a characteristic colony in fresh, pure, 18–24 hour culture.
- d) Rub the swab on the moistened disk so that the growth goes into the filter paper of the disk.
- e) Examine the disk: if the reaction is positive, the areas of the disk containing growth will turn a characteristic red/pink color. Reactions typically occur within five minutes.
- f) Record results. Strains for which the inoculum on the nitrocefin disk turns red/pink are considered “ $\beta$ -lactamase positive”. Strains for which the inoculum on the nitrocefin disk does not change color are considered “ $\beta$ -lactamase negative.”

#### ***Nitrocefin liquid reagent***

If it is anticipated that a large number of isolates will be tested, laboratorians should investigate obtaining nitrocefin powder and preparing the liquid reagent. The nitrocefin test using liquid reagent is performed either by dropping reagent directly on colonies growing on selective or nonselective media, or by diluting the reagent and using it as a suspension medium for bacterial growth in a tube. Although the former method is easier as it involves fewer steps, **the advantage of the latter method is that it uses lesser amounts of the costly liquid reagent.** (Methods for the different preparations of the nitrocefin reagent as used for each of these tests are included in Appendix 2.)

To perform the test for  $\beta$ -lactamase production with liquid nitrocefin reagent using the plate method, use an eyedropper, Pasteur pipette or inoculating loop to

place a drop of the undiluted reagent directly onto fresh gonococcal colonies growing on selective or nonselective culture media. After several minutes, the colonies will turn pink if the gonococcal strain is producing  $\beta$ -lactamase, and should be recorded as “ $\beta$ -lactamase positive.” If, after ten minutes, no color change has occurred on the colonies dropped with reagent, the gonococcal strain is considered “ $\beta$ -lactamase negative,” and should be recorded as such.

To perform the test for  $\beta$ -lactamase production with liquid nitrocefin reagent using the tube method, dispense dilute nitrocefin solution (25 mg/L prepared in 0.1M phosphate buffer) into a test tube, and use it to prepare a heavy suspension (~ McFarland 2) of the suspect gonococcal colonies from 18–24 hour culture. If  $\beta$ -lactamase producing organisms are present, the suspension should change color from colorless/yellow to pink within 15 seconds; record a strain exhibiting this color change as ‘ $\beta$ -lactamase positive.’ If after five minutes no color change has occurred in the suspension, record the strain as ‘ $\beta$ -lactamase negative.’

Results of  $\beta$ -lactamase tests are used in conjunction with results of antimicrobial susceptibility tests performed according to NCCLS methodologies.

### Antimicrobial susceptibility testing of *N. gonorrhoeae* using NCCLS methodologies

Antimicrobial susceptibility testing by both disk diffusion and the antimicrobial gradient strip Etest® method are conducted on the same standardized medium. Because gonococci are fastidious, antimicrobial susceptibility tests for most antimicrobial agents are performed on a GC agar base medium supplemented with IsoVitalEx or an equivalent supplement. **Mueller-Hinton medium, on which susceptibilities of most aerobic bacteria are determined, is not suitable for determining gonococcal susceptibilities;** however, Mueller-Hinton broth can be used to prepare the gonococcal cell suspensions that will be tested. Furthermore, gonococcal susceptibilities should not be determined on media containing chocolate blood or hemoglobin because of the variability of blood products (which may affect susceptibilities of *N. gonorrhoeae* to various antimicrobial agents). Antimicrobial susceptibility test results for *N. gonorrhoeae* should only be interpreted when tested on GC-susceptibility test medium, a standard quality controlled GC agar base medium plus 1% defined supplement.

A sample form for recording the results of antimicrobial susceptibility tests for *N. gonorrhoeae* is included in Figure 27.

### Antimicrobial susceptibility testing of *N. gonorrhoeae* by disk diffusion

Disk diffusion testing should be carried out as defined by the NCCLS performance standards and with the NCCLS quality control strain *N. gonorrhoeae* ATCC 49226. It is recommended that laboratories obtain additional gonococcal reference strains exhibiting resistance patterns not exhibited by ATCC 49226: supplemental QC strains, tested routinely by disk diffusion and agar dilution methods with

FIGURE 27: Sample form for recording antimicrobial susceptibility test results for *Neisseria gonorrhoeae*

<b>Date of Testing:</b> _____/_____/_____ <i>Test performed by:</i> _____		<b>Interpretation of susceptibility: S = susceptible I = intermediate R = resistant</b>									
<b>Specimen number</b>	<b>Antimicrobial: Ciprofloxacin</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>	<b>Antimicrobial:</b>
	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
NCCLS QC strain ATCC 49226 Q/C in range? →	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No
(Other QC strain) <sup>a</sup>											
Q/C in range? →	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No
<sup>a</sup> Choice of supplemental QC strains will depend upon the antimicrobial agents tested, and therefore several QC strains may be indicated.											
<b>Reviewed by:</b> _____ <b>Date of Report:</b> _____/_____/_____											

**Note:** After 20-24 hours of incubation, check the results for the quality control ("QC") strains against the acceptable range of inhibition zone diameters (mm) or MICs (µg/ml); if they are in control, continue reading results for the test isolate. (Inhibition zone ranges and breakpoints for interpretation of results may be found in Tables 9 and 10.)

consistent results, may be obtained from the *Neisseria* Reference Laboratory, Gonorrhea Research Branch, CDC (see Appendix 14). Quality control values for disk diffusion zone diameter sizes for these strains are presented in Table 9.

### Methods

- a) Label one plate of GC-chocolate agar for each clinical isolate and QC strain to be tested.
- b) Inoculate plates with each test strain and streak for isolation. Incubate inoculated plates at 35°–36.5°C in a CO<sub>2</sub>-supplemented atmosphere with increased humidity for 16–20 hours.
  - **Note:** if isolates are maintained in culture prior to inoculation for antimicrobial susceptibility testing, they must be subcultured every 24 hours prior to being tested.
  - **Note:** if isolates are stored frozen prior to inoculation for antimicrobial susceptibility testing, they must be subcultured at least once after initial culture from the frozen preparation prior to being tested.
- c) Suspend isolated colonies (from the overnight cultures prepared in steps a and b) in 1.0–2.0 ml of Mueller-Hinton broth (or PBS). Mix the suspension thoroughly on a vortex mixer to break up clumps of growth as much as possible.
  - It is easier to prepare the suspensions with a swab<sup>28</sup> than with an inoculating loop. The best method to avoid excessive clumping of growth in the suspension is to roll the swab over the colonies rather than to use a scraping method to harvest cells.
- d) Adjust the turbidity of the cell suspension to the turbidity of a 0.5 McFarland standard by comparing tubes against black and white lines and adding broth or culture as needed (see Figures 51 and 52 in Appendix 2). **The suspension must be used to inoculate the plate within 15–20 minutes after preparation, or else it must be discarded** and a new suspension prepared.
  - **Note:** The inoculation step must be completed within 15–20 minutes because the organisms will begin to die within a short time after the suspension is prepared, and even though the suspension will be visually comparable to the McFarland standard, the viability of the inoculum delivered onto the test medium may be too low to produce reliable antimicrobial susceptibility test results.
  - If there are many cultures to test, they should be done in small batches (*e.g.*, five or six isolates at a time) to avoid loss of viability.

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<sup>28</sup> Notes on the survival of *N. gonorrhoeae* with different swab materials are included in Table 29, Appendix 8.

- e) Pour 60 ml of GC base medium containing 1% defined supplement into a 150-mm diameter plate to a uniform depth of 3–4 mm (in order to assure proper conditions for disk diffusion results). The number of plates required for the testing of each strain will be dependent upon the number and type of antimicrobial agents to be tested, as some have larger inhibition zone sizes than others and the zones of inhibition must not overlap. Generally speaking, GC susceptibility tests have no more than 3 disks on each plate.

Plates to be used for antimicrobial susceptibility testing must have warmed to room temperature before they are inoculated with the cell suspension. **The surface of the plate must also be dry prior to inoculation;** if not, invert the plates and dry them with lids slightly open either in an incubator at 35°–36.5°C, or in a biohazard hood. There should be no visible drops of moisture on the surface of the agar when the plates are inoculated.

- f) Moisten a sterile applicator swab<sup>28</sup> in the standardized cell suspension and remove any excess moisture by rotating the swab against the glass above the liquid in the tube. Inoculate the entire surface of each plate three times, rotating the plate 60° each time to ensure confluent growth (Figure 34).
- g) Store the inoculated plates at room temperature for 3–5 minutes to allow the medium to absorb the moisture from the inoculum. **It is essential that the surface of the medium is dry before the antimicrobial disks are applied.** Plates may be dried in an incubator or biohazard hood as described in step e. (If it takes longer than 15 minutes for the inoculum to dry, use a smaller volume / express more suspension from the swab in the future.)
- h) Using sterile forceps, tweezers or a disk dispenser, apply disks of the selected antimicrobial agents to the surface of the inoculated medium; tap them to ensure they are in complete contact with the surface of the agar. **Once a disk has touched the agar surface, diffusion begins and it must not be moved.** All disks should be placed approximately the same distance from the edge of the plate and from each other (Figure 28).
- i) Cover and invert the inoculated plates and incubate them at 35°–36.5°C in a 3%–5% CO<sub>2</sub> atmosphere (in a CO<sub>2</sub>-incubator or candle-extinction jar) for 20–24 hours.
- j) At 20–24 hours after inoculation and incubation, read the results of the antimicrobial susceptibility tests.
- Examine the plates from the back, viewed from the top down against a black background and illuminated with reflected light (so hazy growth is more easily seen). Measure the diameter of the zone of inhibition with calipers, a ruler, or a ruler on a stick (see Figure 6).
  - Read the results for ATCC 49226 and compare them to the values in Table 9; if they are in control, continue reading and comparing results for the other

QC strains tested. If these are also in control, continue to read and record results for the clinical isolates.

- k) Interpret the results. Table 10 presents zone inhibition diameters and equivalent minimal inhibitory concentrations (MICs) for test strains, along with the NCCLS standard interpretations of those zones diameters as sensitive, intermediate, or resistant.

After interpreting results, report them back to the primary laboratory.

### ***Antimicrobial susceptibility testing of *N. gonorrhoeae* by Etest® antimicrobial gradient strip***

Antimicrobial susceptibility testing with the Etest® antimicrobial gradient strip<sup>29</sup> is technically as simple to perform as the disk diffusion test, but provides semi-quantitative MIC results. The strip is impregnated with a standard gradient of antimicrobial agent, and the front of the strip has MIC values that are to be read in correspondence with inhibition of growth on the plate after incubation. **Always read the insert** in the package of Etest® strips, and **follow the manufacturer's instructions** for performance of the test.

Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on GC base medium plus 1% defined growth supplement; methods for the preparation and QC of this medium are included in Appendix 2 ("Media, Reagents and Quality Control"). The standardization of the inoculum and methods for the inoculation of the test plate are the same for the Etest® as they are for the disk diffusion test for *N. gonorrhoeae*; follow steps *a* through *g* above, and then continue with step *h*, below. Strict quality control practices are of extreme importance in order for the proper performance and appropriate interpretation of the antimicrobial susceptibility test. If conditions cannot be controlled and standardized, it is better that the laboratory not perform the antimicrobial susceptibility test at all, because the results obtained cannot be interpreted according to standardized criteria. Inaccurate results are useless to the clinician, can even cause harm to a case-patient, and should not be recorded for use in public health policy treatment decision-making.

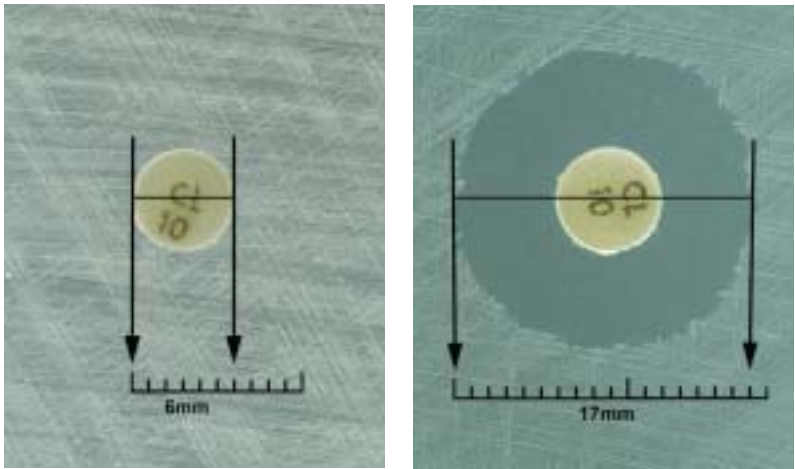
Laboratorians should ensure that the Etest® strips used for antimicrobial susceptibility testing of *N. gonorrhoeae* strains cover the appropriate range of antibiotic concentrations for these organisms.<sup>30</sup>

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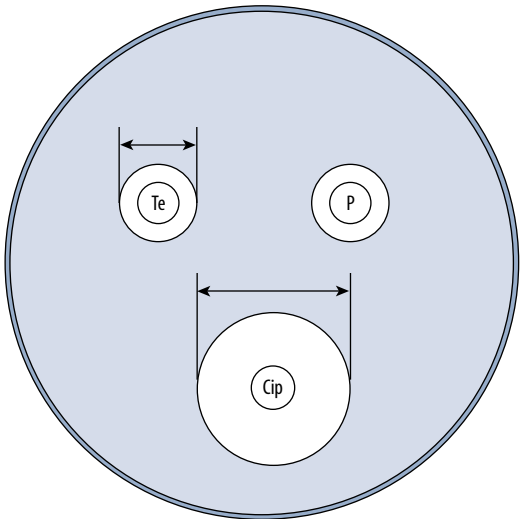
<sup>29</sup> The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor settings (see Appendix 13).

<sup>30</sup> Please note that for certain antimicrobial agents (particularly some  $\beta$ -lactams), the Etest® is available in a high- and low range of concentrations. For testing of *N. gonorrhoeae* with the ceftriaxone Etest®, for example, it is recommended that laboratories use the low-range concentration (0.002  $\mu\text{g/ml}$  – 32  $\mu\text{g/ml}$ ) rather than the high-range concentration (0.016  $\mu\text{g/ml}$  – 256  $\mu\text{g/ml}$ ). A complete list of strips and ranges of concentrations is available from AB Biodisk (at <http://www.abbiobdisk.se/productservice/product.htm> ).

FIGURE 28: Disk diffusion testing: disk placement for *Neisseria gonorrhoeae* and measurements of inhibition zone diameters



Top: Photographs of bacterial growth, zones of inhibition, and measurement of the zones. Note that the disk on the left is surrounded by a resistant strain and the diameter of the zone of inhibition is equivalent to the diameter of the disk (6 mm), whereas the figure on the right shows a strain with a zone of inhibition of 17 mm.



Bottom: The shaded area represents uniform growth of the strain on the plate; the white areas surrounding the disks represent zones of inhibition. Zones of inhibition are measured as indicated by the double-arrow lines.

**Note:** Calipers or a ruler on a stick (see Figure 6) can be helpful for measuring the diameter of a zone of inhibition.

TABLE 9: Acceptable limits for MICs and inhibition zone diameters for quality control strains of *Neisseria gonorrhoeae*

Antimicrobial agent (disk concentration)	Diameter of disk diffusion zone of inhibition (mm) and equivalent minimal inhibitory concentration (MIC) [ $\mu\text{g/ml}$ ] <sup>a</sup>							
	Strain <sup>b</sup>	ATCC 49226 <sup>c</sup>	F-28	P88TE	CDC 10,328	CDC 10,329	SP-15	SP-14
	Phenotype	Susceptible	SpCR	PP-TR	CipI	CipR	"AzrR"	"CrADS"
Penicillin (10-unit)	Disk (mm) [MIC $\mu\text{g/ml}$ ]	26 – 34 [0.25 – 1.0]	37 – 47 [0.015 – 0.06]	6 – 10 [4.0 – 64.0]	<NT> [32.0 – $\geq 64.0$ ] <sup>e</sup>	<NT> [32.0 – $\geq 64.0$ ] <sup>e</sup>	<NT> [0.5 – 1.0]	<NT> [4.0 – 8.0]
Tetracycline (30- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	30 – 42 [0.25 – 1.0]	35 – 40 [0.125 – 0.5]	14 – 19 [8.0 – 32.0]	<NT> [0.5 – 1.0]	<NT> [2.0 – 8.0]	<NT> [1.0 – 4.0]	<NT> [2.0 – 8.0]
Spectinomycin (100- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	23 – 29 [8.0 – 32.0]	6 – 7 [ $\geq 128.0$ ]	22 – 25 [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]
Ceftriaxone (30- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	39 – 51 [0.004 – 0.016]	49 – 62 [0.0005 – 0.004]	43 – 53 [0.002 – 0.008]	<NT> [ $\leq 0.002$ – 0.008] <sup>d</sup>	<NT> [0.004 – 0.015]	<NT> [0.004 – 0.015]	<NT> [0.06 – 0.125]
Cefixime (5- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	37 – 45 [0.004 – 0.03]	<NT> [0.001 – 0.008]	<NT> [0.004 – 0.03]	<NT> [0.008 – 0.06]	<NT> [0.008 – 0.125]	<NT> [0.008 – 0.06]	<NT> [0.25 – 0.5]
Ciprofloxacin (5- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	48 – 58 [0.001 – 0.008]	40 – 55 [ $\leq 0.002$ – 0.008] <sup>d</sup>	45 – 55 [ $\leq 0.001$ – 0.004] <sup>d</sup>	30 – 34 [0.25 – 0.5]	21 – 26 [1.0 – 2.0]	<NT> [0.002 – 0.015]	<NT> [8.0 – 32.0]
Ofloxacin (5- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	43 – 51 [0.004 – 0.016]	40 – 55 [0.004 – 0.015]	40 – 50 [0.004 – 0.015]	27 – 32 [0.25 – 1.0]	18 – 21 [2.0 – 4.0]	<NT> [0.008 – 0.03]	<NT> [<IND>]
Azithromycin (15- $\mu\text{g}$ )	Disk (mm) [MIC $\mu\text{g/ml}$ ]	<NT> [0.125 – 0.5] <sup>c</sup>	<NT> [0.03 – 0.125]	<NT> [0.03 – 0.06]	<NT> [0.03 – 0.06]	<NT> [0.125 – 0.5]	19 – 22 [1.0 – 4.0]	<NT> [0.125 – 0.5]
$\beta$ -lactamase production	(+ / –)	–	–	+	+	+	–	–

<sup>a</sup> These results were developed on a GC susceptibility test medium of GC II agar base medium plus 1% IsoVitalex; the ranges presented here may vary with different formulations of GC agar base and growth supplement.

<sup>b</sup> These QC strains can be obtained from the Gonorrhea Research Branch, CDC. (See Appendix 14 for an address.)

<sup>c</sup> ATCC 49226 is the NCCLS-recommended quality control strain. MICs and inhibition zone diameters for ATCC 49226 are those recommended by the NCCLS, except for MICs for azithromycin which were derived from interlaboratory testing by six laboratories.

<sup>d</sup> Reference laboratories have not tested these QC strains against a concentration of ciprofloxacin lower than the lowest MIC shown.

<sup>e</sup> Reference laboratories have not tested these QC strains against a concentration of penicillin higher than the highest MIC shown.

<NT> indicates not tested by disk diffusion methods; <IND> indicates not tested by MIC methods.

TABLE 10: Interpretive criteria for antimicrobial susceptibility of *Neisseria gonorrhoeae*

Antimicrobial agent	Disk potency	Breakpoints for zone of inhibition (mm) and equivalent MIC (µg/ml) <sup>a</sup>			NCCLS QC strain <i>N. gonorrhoeae</i> ATCC 49226
		Susceptible	Intermediate	Resistant	
Penicillin	10 units	≥ 47 mm (≤ 0.06 µg/ml)	27 – 46 mm (0.125 – 1.0 µg/ml)	≤ 26 mm (≥ 2.0 µg/ml)	26 – 34 mm (0.25 – 1.0 µg/ml)
Tetracycline	30 µg	≥ 38 mm (≤ 0.25 µg/ml)	31 – 37 mm (0.5 – 1.0 µg/ml)	≤ 30 mm (≥ 2.0 µg/ml)	30 – 42 mm (0.25 – 1.0 µg/ml)
Spectinomycin	100 µg	≥ 18 mm (≤ 32.0 µg/ml)	15 – 17 mm (64.0 µg/ml)	≤ 14 mm (≥ 128.0 µg/ml)	23 – 29 mm (8.0 – 32.0 µg/ml)
Ceftriaxone **	30 µg	≥ 35 mm (≤ 0.25 µg/ml)	** **	** **	39 – 51 mm (0.004 – 0.016 µg/ml)
Cefixime **	5 µg	≥ 31 mm (≤ 0.25 µg/ml)	** **	** **	37 – 45 mm (0.004 – 0.03 µg/ml)
Ciprofloxacin	5 µg	≥ 41 mm <sup>b</sup> (≤ 0.06 µg/ml)	28 – 40 mm (0.125 – 0.5 µg/ml)	≤ 27 mm (≥ 1.0 µg/ml)	48 – 58 mm (0.001 – 0.008 µg/ml)
Ofloxacin	5 µg	≥ 31 mm (≤ 0.25 µg/ml)	25 – 30 mm (0.5 – 1.0 µg/ml)	≤ 24 mm (≥ 2.0 µg/ml)	43 – 51 mm (0.004 – 0.016 µg/ml)

\*\* Only “susceptible” interpretive criteria are available for zones and MICs for ceftriaxone and cefixime; isolates with ranges outside the values in this table should be noted as having “decreased susceptibility” and sent to an international reference laboratory for further testing.

<sup>a</sup> Source: NCCLS (2002) *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, PA 19087-1898 USA.

<sup>b</sup> Recent experience has shown that some gonococcal isolates with ciprofloxacin zone sizes of 36 mm (and therefore classified as “intermediate” by current NCCLS criteria) have MICs of 0.06 mg/ml and are classified as “susceptible” by current NCCLS criteria for MICs determined by agar dilution susceptibility testing. More research is needed to clarify the relationship between an MIC of 0.06 µg/ml of ciprofloxacin and the corresponding disk diffusion zone inhibition diameters exhibited by such organisms. It is therefore advised that the antimicrobial susceptibilities of isolates exhibiting inhibition zone diameters of 36–41 mm be confirmed by MIC testing before they are classified as exhibiting intermediate resistance to ciprofloxacin.

## Methods

- a - g) Methods for the preparation of the standard inoculum and the inoculation of the test plates are included in steps *a* through *g* of ‘disk diffusion methods’, listed above.
- h) Remove the Etest® strips from the freezer, and allow them to reach room temperature (approximately 30 minutes). It is extremely important to **keep the Etest® strips that are not going to be used in a freezer at -20°C**.
- i) When the surface of the plate is dry, place the Etest® strips on the agar surface with sterile forceps, tweezers or test-dispenser, as illustrated in Figure 7. (Make sure that the printed MIC values are facing upward, *i.e.*, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.) Once the test strip has touched the surface, it should not be moved.

- Although the manufacturer's insert for the Etest® says that up to two strips can be used on a 100-mm plate and up to six on a 150-mm plate, because gonococci can have such wide zones of inhibition, **this laboratory manual advises using only one Etest® strip per 100 mm plate for *N. gonorrhoeae***. The number of strips on a 150-mm plate will be determined by the combination of drugs being tested; zones of inhibition *must not* overlap. (Once laboratorians have determined the range of susceptibilities of local gonococcal isolates to various antimicrobial agents with the Etest® on 100-mm plates, they can assess which combinations of antimicrobial agents can be tested on a 150-mm plate without overlapping zones of inhibition, usually 3 or 4 antimicrobial agents.)
- j) Incubate the inoculated Etest® plate according to the manufacturer's instructions (usually 20–24 hours at 35°–36.5°C in a 5% CO<sub>2</sub> atmosphere).
  - k) After incubation for 20–24 hours, there will be an ellipse of inhibition of bacterial growth on the plate around the Etest® strip, and the MIC values can be read (see Figure 8).
    - The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (*i.e.*, the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of a gonococcal isolate to ciprofloxacin, an MIC recorded from the graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml and the organism would be interpreted as exhibiting intermediate resistance to ciprofloxacin.
  - l) Read the results for ATCC 49226 and compare them to the values in Table 9; if they are in control, continue reading and comparing results for the other QC strains tested. If these are also in control, continue to read and record results for the clinical isolates. **It is essential to review the MIC results of the quality control strains prior to interpreting the MICs of the clinical isolates.**
  - m) Read and interpret the results for the test strains. Table 10 presents the NCCLS interpretive criteria (susceptible, intermediate, resistant) for different antimicrobials, including those currently recommended for the primary therapy of uncomplicated gonorrhea.

A reading guide for interpretation of Etest® antimicrobial susceptibility results and guidance in reading MICs from the Etest® strip is presented in Figure 8. The guide, included with the permission of AB Biodisk, shows how growth appears around the strip and provides guidance for how the test should be interpreted.

TABLE 11: MIC critical values for *Neisseria gonorrhoeae* and appropriate laboratory response

Antimicrobial agent	Therapeutic dose	Critical MIC value (disk diffusion zone size)	Phenotypic 'resistance' category	Response <sup>a</sup>
Ceftriaxone	125 mg, single dose, intramuscular (IM)	MIC > 0.25 µg/ml (<35 mm)	Decreased susceptibility ( <i>CroDS</i> )	Isolates showing higher MICs or smaller zone sizes than these ranges should be retested to confirm the results, preferably with QC strains specifically for the antimicrobial agent showing abnormally high values.
	400 mg, single dose, oral	MIC > 0.25 µg/ml (<37 mm)	Decreased susceptibility ( <i>CfoDS</i> )	
Ciprofloxacin	500 mg, single dose, oral	MIC 0.125 – 0.5 µg/ml (28 – 40 mm)	Intermediate resistance ( <i>CipI</i> )	A confirmed MIC value greater than or equal to the critical MIC should alert laboratorians and clinicians to ascertain that infections caused by such strains (and treated with the WHO-recommended dose of the corresponding agent) responded successfully to therapy.  If, after re-testing, the high MIC is confirmed, an international reference laboratory should be notified and arrangements made to send the isolate for further confirmation.
		MIC ≥ 1.0 µg/ml (≤ 27 mm)	Resistance ( <i>CipR</i> )	
Ofloxacin	400 mg, single dose, oral	MIC 0.5 – 1.0 µg/ml (25 – 30 mm)	Intermediate resistance ( <i>OfxI</i> )	
		MIC ≥ 2.0 µg/ml (≤ 24 mm)	Resistance ( <i>OfxR</i> )	
Azithromycin	2 g, single dose, oral	MIC ≥ 1.0 µg/ml <sup>b</sup> (≤ 25 mm) <sup>b</sup>	'Resistance' <sup>b</sup> ( <i>AzmR</i> )	
Spectinomycin	2 g, single dose, intramuscular (IM)	MIC ≥ 128.0 µg/ml (≤ 14 mm)	Resistance ( <i>SpcR</i> )	

<sup>a</sup> If MICs greater than or equal to the critical MIC have already been confirmed in a region, it may be desirable to confirm the MIC with regard to evaluating treatment outcome for patient management purposes; however, it is not necessary to re-contact an international reference laboratory.

<sup>b</sup> NCCLS [2002] has not recommended criteria for the interpretation of susceptibilities of *N. gonorrhoeae* to azithromycin. Because there are limited outcome data corresponding to treatment of gonococcal infections with azithromycin (2 g), it is suggested that laboratorians and clinicians collaborate to correlate treatment outcome data with *in vitro* susceptibility results to establish an interpretive breakpoint for resistance corresponding to a 5% treatment failure rate. In the absence of conclusive clinical outcome data, this document presents a 'working' interpretive criterion for resistance based on a limited number of treatment failures and laboratory *in vitro* agar dilution and disk diffusion antimicrobial susceptibility data [Handfield *et al.* 1994; CDC, unpublished data]: *N. gonorrhoeae* strains with a confirmed MIC of ≥ 1.0 µg/ml exhibit an *in vitro* 'resistant' phenotype to azithromycin.

- **Note:** Although the WHO-recommended dose for treatment of uncomplicated gonorrhea with azithromycin is 2 grams in a single oral dose, because a 1-gram single oral dose of azithromycin is recommended for the treatment of genital *Chlamydia trachomatis* infections, this dose may be used incidentally to treat gonococcal co-infections. Evaluation of clinical treatment outcomes has indicated that gonococcal infections caused by strains with MICs of ≥ 0.125 µg/ml may fail to respond to treatment with the 1-gram dose of azithromycin [Young *et al.* 1997].

When reading any antimicrobial susceptibility test results for *N. gonorrhoeae*, the laboratorian should be aware of critical values that indicate a need for retesting. Table 11 presents a listing of critical antimicrobial susceptibility test values for the laboratory to be aware of. If the MIC results for an organism are higher than those listed for the specific antimicrobial agent, the reference laboratory should re-test the isolate. If the results are still atypical, confirm the identification of the organism, ensure the test is being performed properly, and then re-test the isolate again. If it still produces a high MIC, notify the national and an international reference laboratory and send the isolate for further investigation. Instructions for the preservation and storage of isolates are presented in Appendix 11, while Appendix 12 includes the instructions for regulation-compliant packaging and shipping of isolates.

When the susceptibility value is confirmed upon re-test, the confirmatory laboratory should notify the submitting laboratory and then other laboratories in the regional and international network. If the isolate represents a new antimicrobial resistance phenotype, it is important that the confirming reference laboratory disseminate preserved cultures of the isolate to other reference laboratories for inclusion among susceptibility quality control strains. Isolates showing a previously undescribed resistance pattern should not be used for scientific research (such as the determination of the resistance mechanisms) without permission from the originating clinician and/or laboratory.

## Data for decision-making

Antimicrobial susceptibility testing can be performed on an isolate presumptively identified as *N. gonorrhoeae*, although confirmatory testing should be completed before antimicrobial susceptibility test results exceeding the critical MIC values are reported. (For example, before reporting results, laboratories should confirm the identification of an organism showing an unexpectedly high MIC to, e.g., ceftriaxone.) Once the laboratory has determined the antimicrobial susceptibilities, the information should be reported back to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- Laboratories should screen and report values for antimicrobial agents currently in use for primary therapy of gonorrhea in the region and, ideally, also for the second line drugs.
- MIC “critical values” can be useful tools to initiate (enhanced) surveillance and epidemiological investigations to determine if there is an association between the *in vitro* susceptibility of a strain and the clinical outcome.
- Extended-spectrum cephalosporins, fluoroquinolones, and spectinomycin are recognized as the most effective antimicrobial agents for the treatment of gonorrhea in most geographic areas of the world.

- The antimicrobial agent and dose chosen should be effective against at least 95% of local gonococcal strains.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be readily available.
- It should be possible to store the chosen antimicrobial agent under conditions (e.g., refrigeration) that will maintain the activity of the drug.

It is important to consider the above factors when making decisions relating to treatment of gonorrhea. Determination of antimicrobial susceptibilities to therapeutic agents will help public health officials review the appropriateness of treatment recommendations for local populations, and surveillance of antimicrobial susceptibilities will promote effective disease control.

# Bacterial Agents of Enteric Diseases of Public Health Concern

*Salmonella* serotype Typhi

*Shigella*

*Vibrio cholerae*

## *Salmonella* serotype Typhi

### IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

*Salmonella* serotype Typhi (*S. Typhi*), the etiologic agent of typhoid fever, causes an estimated 16.6 million cases and 600,000 deaths worldwide each year. A syndrome similar to typhoid fever is caused by “paratyphoid” serotypes of *Salmonella*. The paratyphoid serotypes (*i.e.*, *S. Paratyphi* A, *S. Paratyphi* B, and *S. Paratyphi* C) are isolated much less frequently than *S. Typhi*. Rarely, other serotypes of *Salmonella*, such as *S. Enteritidis*, can also cause “enteric fever.” Like other enteric pathogens, *S. Typhi* is transmitted through food or water that has been contaminated with feces from either acutely infected persons, persistent excretors, or from chronic asymptomatic carriers. Humans are the only host for *S. Typhi*; there are no environmental reservoirs.

Effective antimicrobial therapy reduces morbidity and mortality from typhoid fever. Without therapy, the illness may last for 3–4 weeks and case-fatality rates may exceed 10%. With appropriate treatment, clinical symptoms subside within a few days, fever recedes within 5 days, and mortality is reduced to approximately 1%. Relapses, characterized by a less severe but otherwise typical illness, occur in 10%–20% of patients with typhoid fever, usually after an afebrile period of 1–2 weeks. Relapses may still occur despite antimicrobial therapy.

*S. Typhi* is most frequently isolated from blood during the first week of illness, but it can also be present during the second and third weeks of illness, during the first week of antimicrobial therapy, and during clinical relapse. Fecal cultures are positive in approximately half the cases during the first week of fever, but the largest number of positive cultures occurs during the second and third weeks of disease. Bone marrow cultures are frequently positive (90% of cases) and are more likely to yield *S. Typhi* than are cultures from any other site, especially when the patient has already received antimicrobial therapy. Organisms can also be isolated from duodenal aspirates, rose spots, and infrequently (*i.e.*, in approximately 25% of cases) from urine cultures.

In typhoid fever, serologic responses to O, H, and Vi antigens usually occur by the end of the first week of illness. The Widal test, which measures antibody responses to H and O antigens, can suggest the diagnosis, but the results are not definitive and must be interpreted with care because titers also may be elevated in response to a number of other infections. High-titer, single serum specimens from adults living in areas of endemic disease have little diagnostic value. Even when paired sera are used, the results must be interpreted in light of the patient's history of typhoid immunization and previous illness, the stage of the illness when the first serum specimen was obtained, the use of early antimicrobial therapy, and the reagents used.

There are currently (2002) at least two effective vaccines available for typhoid, both of which were recently licensed for use in the United States. The oral live attenuated vaccine (for use in children aged 6 years and older) and the parenteral (*i.e.*, injectable) capsular polysaccharide vaccine (for use in children aged 2 years and older) each have efficacy of 50%–80% and fewer adverse events associated with their use than earlier typhoid vaccines. A team doing research in Vietnam reported promising preliminary success of a new conjugate vaccine in early 2001. The two U.S.-licensed vaccines have been widely and effectively used by travelers to typhoid-endemic regions, though the expense and limited experience with their use as a public health intervention in countries with high endemic rates of typhoid fever precludes the widespread use of these vaccines in countries with limited resources. Nonetheless, it is good policy for laboratory technicians who may be working with this organism to supplement their laboratory safety practices and ensure that their vaccination status against typhoid fever remains current.

In developing countries, typhoid fever is frequently diagnosed solely on clinical grounds; however, isolation of the causative organism is necessary for a definitive diagnosis. Isolation of the agent is also a necessity for the performance of antimicrobial susceptibility testing.

Resistance to the antimicrobial agents amoxicillin, trimethoprim-sulfamethoxazole, and chloramphenicol is being increasingly reported among *S. Typhi* isolates; quinolone resistance has been reported from the Indian subcontinent and Southeast Asia. **Determining antimicrobial resistance patterns is essential in recommending treatment.** In areas where resistance to these agents is common among circulating *S. Typhi* strains, fluoroquinolones and parenteral third-generation cephalosporins are probably the best choice for empiric treatment of typhoid fever. Cefixime may be recommended in some cases as a less expensive, oral alternative to parenteral ceftriaxone.

## Identification of *S. Typhi*

A preliminary report of typhoid can be issued to a clinician as soon as a presumptive identification of *S. Typhi* is obtained. Methods for the isolation of

*S. Typhi* from normally sterile sites (e.g., blood, bone marrow, and urine) are presented in Appendix 3; isolation of *S. Typhi* from fecal specimens is presented in Appendix 10. Blood, bone marrow, or urine specimens collected from a patient with suspect typhoid fever or a diagnosis of fever of unknown origin and sent to a laboratory should be cultured on blood or chocolate agar; in addition, if resources permit the use of more than one medium, MacConkey agar (MAC) should be inoculated. Fecal specimens should be cultured on selective agar media (e.g., bismuth sulfite agar [BS] or desoxycholate citrate agar [DCA]). Isolates from blood, bone marrow or urine should be Gram stained, whereas isolates obtained from stool specimens should not. In most situations, presumptive identification is based on the reaction of the isolate on Kligler iron agar (KIA) / triple sugar iron Agar (TSI) **and** a positive serologic reaction in *Salmonella* Vi or D antisera.

If gram-negative rods are cultured from specimens obtained from normally sterile sites and/or their culture yields colorless colonies on MAC, the laboratorian should inoculate KIA/TSI. Isolates that have a reaction typical of *S. Typhi* on KIA/TSI should then be tested with Vi and D antisera. The results of the serologic testing should be promptly reported to health authorities, and Mueller-Hinton agar should be inoculated for antimicrobial susceptibility testing. For **any** blood isolate, antimicrobial susceptibility testing should not be delayed pending biochemical or serologic identification.

Although clinicians will not necessarily be waiting for the results of antimicrobial susceptibility tests or even the verification of identification, the reference laboratory should confirm the pathogen's identification via biochemical and serologic characterization and record these and the antimicrobial susceptibility results along with the patient's demographic information for epidemiologic purposes. A flowchart of tests for the identification of an agent as *S. Typhi* is presented in Figure 29, and Figure 30 illustrates a worksheet to record laboratory data.

### Kligler iron agar and triple sugar iron agar

Suspicious colonies should be carefully picked from plating media to a screening medium such as Kligler iron agar (KIA) or triple sugar iron agar (TSI) or to any nonselective agar medium and then incubated overnight. Select at least one of each colony type of the well-isolated colonies on each plate. Using an inoculating needle, lightly touch **only the center of the colony**. Do not take the whole colony or go through the colony and touch the surface of the plate because this practice could result in picking up contaminants that may be present on the surface of the agar. If the ability to select an isolated, pure colony is questionable, the suspicious colony should be purified by streaking for isolation on another agar plate before inoculating the colony to a TSI/KIA slant.

TSI and KIA are inoculated by stabbing the butt and streaking the surface of the slant. The caps should be loosened before incubation. After incubation for 24

FIGURE 29: Flowchart for the isolation and identification of *Salmonella* ser. Typhi

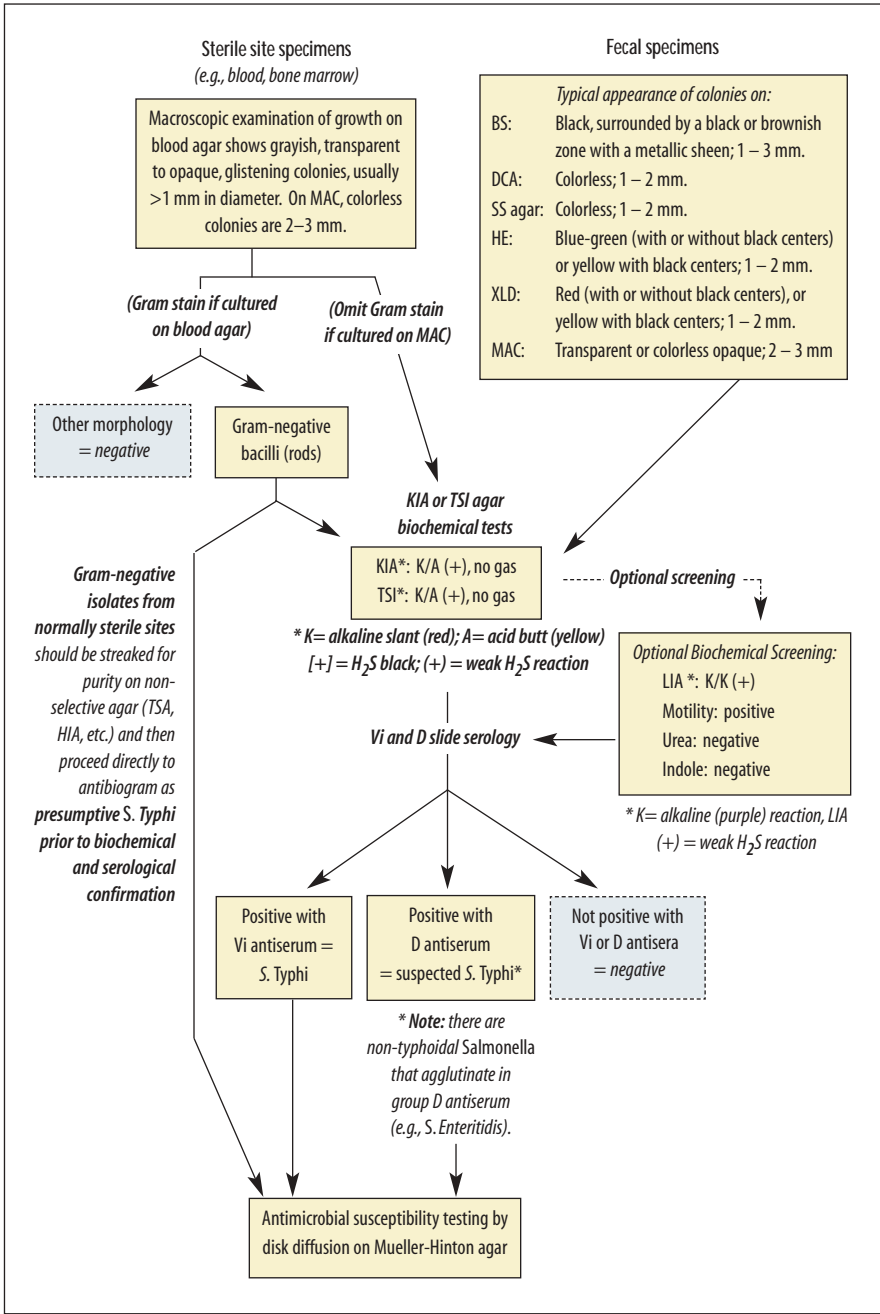


FIGURE 30: Sample worksheet for *Salmonella* ser. Typhi test results

Specimen number	Agar medium <sup>a</sup>	Colony <sup>b</sup>	Gram stain <sup>c</sup>	KIA / TSI	OPTIONAL					Slide Serology <sup>d</sup>		Identification
					LIA	Motility	Urea			VI	D	
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										
		1										
		2										
		3										

<sup>a</sup> The choice of agar medium will depend on whether a specimen is from a normally sterile site (e.g., blood, bone marrow, urine) or is a fecal specimen. Sterile site specimens should be cultured on blood agar; fecal specimens should be plated on selective media (such as BS, DCA, SS, HE, XLD, or MAC).

<sup>b</sup> If macroscopic examination of morphology reveals there is more than one type of colony on a plate, perform tests to identify each of the different isolates.

<sup>c</sup> Gram stain should only be performed on isolates from normally sterile sites (e.g., blood, bone marrow, urine) and should not be performed on growth from MAC or other selective media.

<sup>d</sup> If prompt identification is required for clinical decision-making, slide serology may precede biochemical testing.

hours at 35°–37°C, the TSI or KIA slants are observed for reactions typical of *Salmonella*. **On TSI or KIA slants, *S. Typhi* characteristically produces an alkaline slant (red, “K”), an acid butt (yellow, “A”), and a small amount of blackening of the agar (H<sub>2</sub>S, +) at the site of the stab on the slant and in the stab line (Figure 31); no gas (G) is produced.** It is worth noting that occasionally *S. Typhi* isolates do not produce H<sub>2</sub>S. *S. Paratyphi A* isolates on TSI/KIA are usually K/AG and do not produce H<sub>2</sub>S. Most other *Salmonella* serotypes produce a K/AG+ reaction, indicating that glucose is fermented with gas and H<sub>2</sub>S production. Table 12 summarizes the reactions of *Salmonella* on screening biochemicals.

### Additional screening biochemicals for the identification of *S. Typhi*

Isolates can be identified biochemically as *Salmonella* by traditional tubed media or commercial biochemical systems. Table 12 lists biochemical reactions of the tests that are helpful screening tests for *S. Typhi*. After performing the tests, read and record the results, then compare them to results for presumptive *S. Typhi*. If they match, then proceed by confirming with serologic testing if it has not already been performed.

#### ***Lysine iron agar***

Lysine iron agar (LIA) is a useful screening medium because most *Salmonella* isolates decarboxylate lysine and produce H<sub>2</sub>S, whereas gas production varies by serotype. Preparation and quality control (QC) of this medium are described in Appendix 2. Inoculate LIA by stabbing the butt and streaking the surface of the slant; read and interpret the reactions after incubation for 24 hours at 35°–37°C for 24 hours.

On LIA, *Salmonella* typically give an alkaline (purple) reaction on the slant and butt and may produce gas and H<sub>2</sub>S (blackening of medium) as well, as indicated in Table 12. When the reaction in the butt of the tube is alkaline, the lysine is decarboxylated and the isolate is termed “lysine-positive.” Unlike most other *Salmonella*, *S. Paratyphi A* isolates are lysine-negative and appear yellow on LIA.

If a diagnosis of infection with *S. Typhi* is suspected and prompt diagnosis is required to identify appropriate treatment, suspect isolates should be screened with antisera prior to biochemical identification. However, in the setting of a public health study, because slide serology can be performed using growth from KIA, TSI, or LIA, performing serology after those tests and saving antiserum for only those isolates showing biochemical characteristics typical of *S. Typhi* is more cost-effective.

#### ***Motility agar***

Motility agar should be inoculated with a straight inoculating needle, making a single stab about 1–2 cm down into the medium. The surface of the motility agar

FIGURE 31: *Salmonella* ser. Typhi colonies on triple sugar iron (TSI) Agar



On triple sugar iron agar (TSI) or Kligler iron agar (KIA) slants, *S. Typhi* characteristically produces an alkaline slant (red, "K"), an acid butt (yellow, "A"), and a small amount of blackening of the agar ( $H_2S$ , +) at the site of the stab on the slant and in the stab line; no gas (G) is produced.

should be dry when used: moisture can cause a non-motile organism to grow down the sides of the agar creating a haze of growth and appearing to be motile. Motility agar may be inoculated with growth from a KIA or TSI that shows a reaction typical of *S. Typhi*. Alternatively, motility agar can be inoculated at the same time as the KIA or TSI slant by using the same inoculating needle without touching the colony again. (When motility agar is to be inoculated at the same time as KIA or TSI, use the same colony to first inoculate the motility agar and then to inoculate the KIA or TSI by stabbing the butt and then streaking the surface of the slant. **Do not select a second colony to inoculate the KIA or TSI after the motility agar has been inoculated because it may represent a different organism.**

TABLE 12. Typical reactions of *Salmonella* spp. in screening biochemicals

Screening medium	<i>Salmonella</i> Typhi	<i>Salmonella</i> Paratyphi A	Nontyphoidal <i>Salmonella</i> or <i>Salmonella</i> Paratyphi B or C
Triple sugar iron agar (TSI)	K/A(+) <sup>a</sup>	K/AG- <sup>a</sup>	K/AG+ <sup>a</sup>
Kligler iron agar (KIA)	K/A(+) <sup>a</sup>	K/AG- <sup>a</sup>	K/AG+ <sup>a</sup>
Lysine iron agar (LIA)	K/K(+) <sup>b</sup>	K/AG- <sup>b</sup>	K/K+ <sup>b</sup>
Hydrogen sulfide (H <sub>2</sub> S)	(weak) <sup>c</sup>	negative	positive
Urea	negative	negative	negative
Motility	positive <sup>c</sup>	positive	positive
Indole	negative	negative	negative

<sup>a</sup> for KIA / TSI: K = alkaline (red); A = acid (yellow); G = gas production; + = black H<sub>2</sub>S produced (weak); – = no H<sub>2</sub>S  
<sup>b</sup> for LIA: K = alkaline (purple); A = acid (yellow); G = gas production; + = black H<sub>2</sub>S produced (weak); – = no H<sub>2</sub>S  
 ~ An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated.  
 ~ An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.  
<sup>c</sup> this reaction occurs 97% of the time.

Examine after overnight incubation at 35°–37°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39). Non-motile organisms do not grow out from the line of inoculation. Motility reactions may be difficult for inexperienced laboratorians to read; therefore, reactions should be compared with positive and negative control strains. *S. Typhi* is usually motile (+ 97%).

Sulfide-indole-motility medium is a combination medium that is commercially available in dehydrated form (see Appendix 2, “Media, Reagents, and Quality Control”). It can be used in place of motility medium.

### Urea medium

Urea medium screens out urease-producing organisms (*e.g.*, *Klebsiella* and *Proteus*). Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35°–37°C. **Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color** (Figure 40). Urease-negative organisms do not change the color of the medium, which is a pale yellowish-pink. *S. Typhi* is always urease negative.

### Slide serology for *S. Typhi* identification

TSI/KIA cultures that are suspicious for *S. Typhi* should be screened serologically with *Salmonella* Vi and group D “O” antisera. Because Vi is a capsular antigen, if it is present, it may mask the somatic “O” group reaction. Therefore, *S. Typhi* isolates will usually either be positive in the Vi or the D antisera (though it is possible they

will be weakly positive in both). Occasionally, *S. Paratyphi C* will also be positive in Vi antiserum, but because it produces gas from glucose and is H<sub>2</sub>S positive, reactions on KIA/TSI allow for the differentiation of *S. Paratyphi C* from *S. Typhi*.

Serologic agglutination tests may be performed in a petri dish or on a clean glass slide.

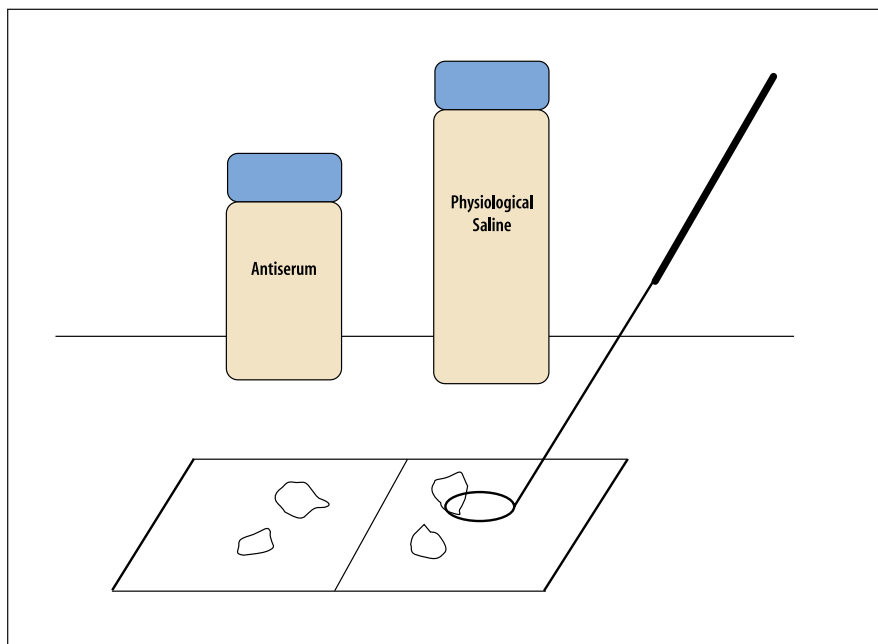
- a) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, LIA, or other nonselective agar medium. Serologic testing should not be done on growth from selective media (e.g., MAC, DCA, BS, or XLD) because selective media may yield false-negative serologic results.
- b) Emulsify the growth in three small drops of physiological saline and mix thoroughly.
- c) Add a small drop of O group D antiserum to one of the suspensions and a small drop of Vi antiserum to a second. The third suspension is used as a control for autoagglutination (roughness). Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 ml can be used. A bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32).
- d) Mix the suspension and antiserum thoroughly and then tilt the slide back and forth to observe for agglutination. It will be easier to see the agglutination if the slide is observed under a bright light and over a black background; if the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping caused by autoagglutination. If autoagglutination occurs, the culture is termed “rough” and cannot be serotyped. Strong agglutination reactions are read as positive.

Cultures that have a TSI/KIA reaction typical of *S. Typhi* and that react serologically in either the Vi or the D antisera can be presumptively identified as *S. Typhi*. The tube agglutination for the “d” flagellar antigen or further biochemical tests may be conducted by reference laboratories to confirm the identification as *S. Typhi*.

## Antimicrobial susceptibility testing of *S. Typhi*

Treatment with an appropriate antimicrobial agent is crucial for patients with typhoid. Because recent reports have noted an increasing level of resistance to one or more antimicrobial agents in *S. Typhi* strains, isolates should undergo antimicrobial susceptibility testing as soon as possible. The disk diffusion method

FIGURE 32: Use of a bent loop to dispense small amounts of antiserum for slide agglutination tests



presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),<sup>31</sup> and, if performed precisely according to the protocol below, will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. However, any deviation from the method can invalidate the results. For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing. Antimicrobial agents suggested for use in antimicrobial susceptibility testing of *S. Typhi* are listed in Table 13.

#### *Special considerations for antimicrobial susceptibility testing of S. Typhi*

As previously mentioned, testing some bacteria against certain antimicrobial agents may yield misleading results because these *in vitro* results do not necessarily correlate with *in vivo* activity. *Salmonella* (including ser. Typhi) isolates, for instance, are usually susceptible to aminoglycosides (e.g., gentamicin, kanamycin) and first- and second-generation cephalosporins using the disk diffusion test, but treatment with these drugs is often not effective [NCCLS 2002].

<sup>31</sup> Formerly known as the “National Committee for Clinical Laboratory Standards,” NCCLS is now known solely by its acronym.

TABLE 13: Antimicrobial agents suggested for use in antimicrobial susceptibility testing of *Salmonella* ser. Typhi

Antimicrobial agents for susceptibility testing of <i>Salmonella</i> serotype Typhi
Ampicillin
Chloramphenicol
Trimethoprim-sulfamethoxazole (cotrimoxazole)
Nalidixic acid*
<i>*If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.</i>

It is also worth noting that **sometimes the result of one antimicrobial susceptibility test will indicate the need for additional tests** to confirm an expected result. For example, when an isolate of *S. Typhi* is resistant to nalidixic acid, it will usually exhibit reduced susceptibility to ciprofloxacin; this scenario may translate in the clinical setting to need for a longer course of treatment. Isolates exhibiting resistance to nalidixic acid should be tested for susceptibility to ciprofloxacin.

Agar disk diffusion testing of *S. Typhi*

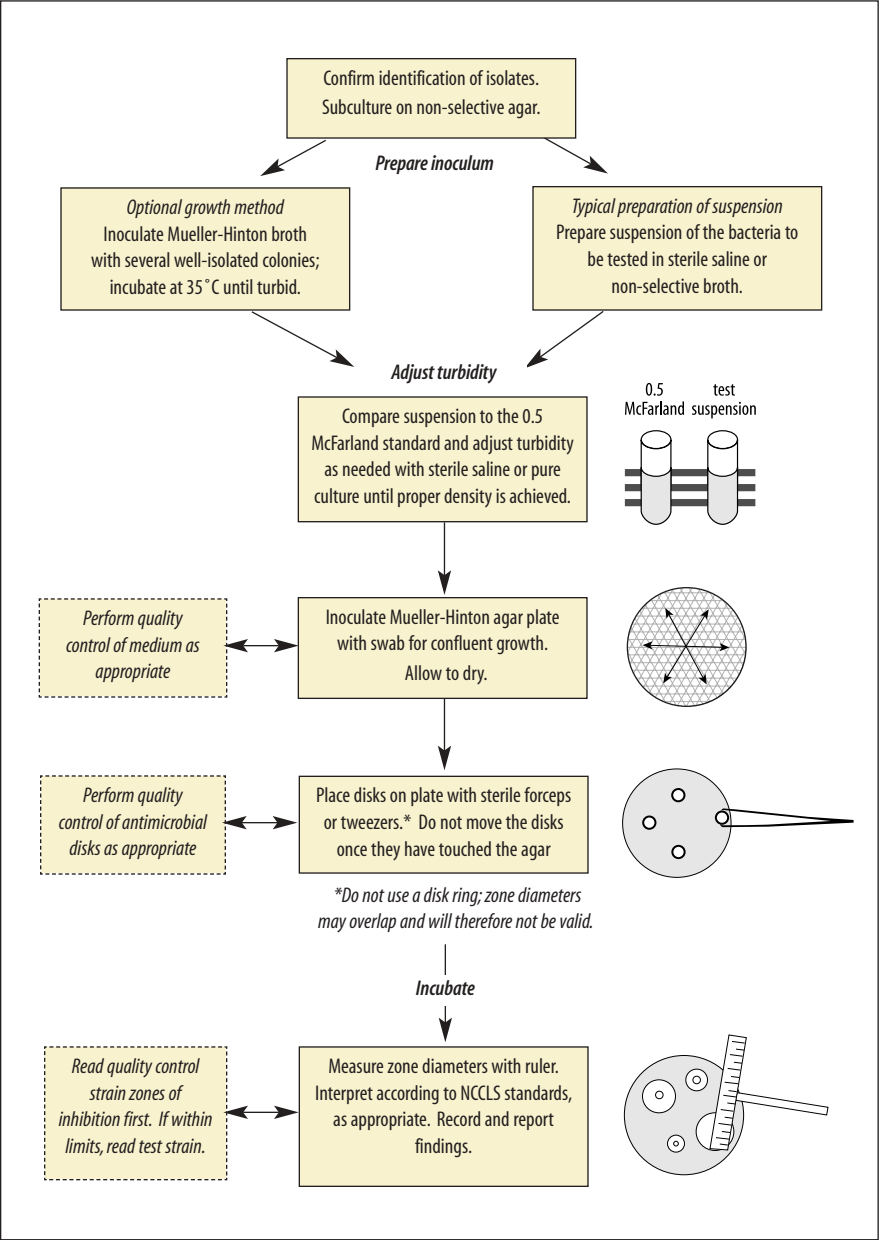
Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2. A summary of the disk diffusion method of antimicrobial susceptibility testing is presented in Figure 33.

A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (see Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

*Preparation of inoculum*

Each culture to be tested should be streaked onto a non-inhibitory agar medium (e.g., blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35°C overnight, select four or five well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (e.g., Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. The bacterial

FIGURE 33: Flowchart of the general procedure for antimicrobial susceptibility testing by disk diffusion



suspension should then be compared to the 0.5 McFarland turbidity standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (see Appendix 2, Figures 51 and 52). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35°C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

### ***Inoculation procedure***

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

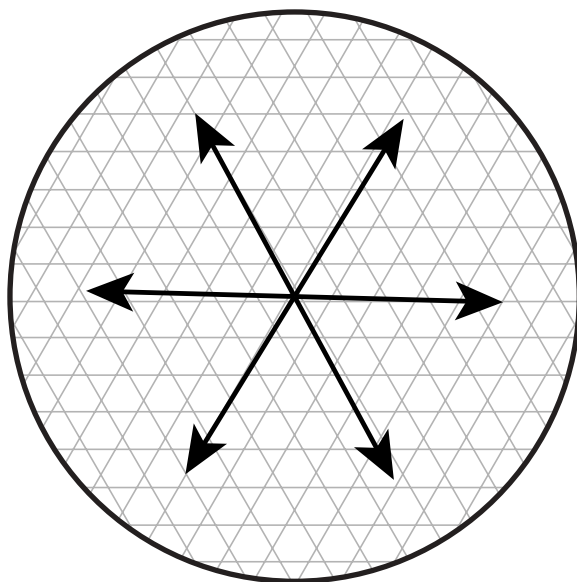
If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture, as may occur when working with cultures from stool specimens), laboratorians may choose to prepare a purity plate to ensure the suspension used for antimicrobial susceptibility testing is pure. To prepare the purity plate, after inoculating the Mueller-Hinton agar plate for confluent growth, label (a portion of) a separate TSA plate (or other non-selective medium) and **use the same swab** of suspension with which the Mueller-Hinton was inoculated to streak for isolation; do not place the swab back into the suspension. Several inocula can be streaked on different sections of a properly labeled purity plate, but the streaks **must not overlap**.

### ***Quality control***

To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing *S. Typhi* and other *Enterobacteriaceae*.) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 14 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a

FIGURE 34: Inoculation of Mueller-Hinton medium for antimicrobial susceptibility tests



Inoculate a Mueller-Hinton plate by dipping a sterile swab into the standardized inoculum one time and then swabbing the entire surface of the medium three times, rotating the plate after each application to ensure an even inoculum for confluent growth.

source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks, even when the isolates being tested are susceptible.

TABLE 14: Inhibition zone diameter size interpretive standards for *Enterobacteriaceae* (for selected antimicrobial disks appropriate for *Salmonella* ser. Typhi)

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)			NCCLS QC strain <i>E. coli</i> ATCC 25922
		Susceptible	Intermediate	Resistant	
Ampicillin	10 µg	≥ 17 mm (≤ 8 µg/ml)	14 – 16 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	16 – 22 mm (2–8 µg/ml)
Chloramphenicol	30 µg	≥ 18 mm (≤ 8 µg/ml)	13 – 17 mm (16 µg/ml)	≤ 12 mm (≥ 32 µg/ml)	21 – 27 mm (2–8 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm (≤ 2/38 µg/ml)	11 – 15 mm (4/76 µg/ml)	≤ 10 mm (≥ 8/152 µg/ml)	23 – 29 mm (≤ 0.5/9.5 µg/ml)
Nalidixic acid	30 µg	≥ 19 mm (≤ 8 µg/ml)	14 – 18 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	22 – 28 mm (1–4 µg/ml)
Ciprofloxacin	5 µg	≥ 21 mm (≤ 1 µg/ml)	16 – 20 mm (2 µg/ml)	≤ 15 mm (≥ 4 mg/ml)	30 – 40 mm (0.004–0.016 µg/ml)

Source: NCCLS (2002) *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, PA 19087 USA.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

**Antimicrobial disks**

The working supply of antimicrobial disks should be stored in a refrigerator (4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate; this reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use.

Apply the antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. The plate surface should be dry, with no liquid remaining. Place the disks individually with sterile forceps or with mechanical dispensing apparatus, and then gently press down onto the agar. In general, no more than 12 disks should be placed on a 150-mm plate and **no more than four disks should be placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved.**

### **Recording and interpreting results**

After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours; if a purity plate was prepared, incubate it under the same conditions. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 28) and record it in millimeters. (A sample worksheet is included in Figure 35.) The measurements can be made with calipers or a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone; in this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. For *S. Typhi*, zones of growth inhibition should be compared with the zone-size interpretative table for *Enterobacteriaceae* (Table 14), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 35). If there is both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

- a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)
- b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).
- c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

### **Data for decision-making**

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of *S. Typhi* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

FIGURE 35. Sample form for recording antimicrobial susceptibility test results for *Salmonella* ser. Typhi

Date of Testing: _____ / _____ / _____		Interpretation of susceptibility: S = susceptible I = intermediate R = resistant					
Test performed by: _____							
Specimen number	Ampicillin	Chloramphenicol	Trimethoprim-sulfamethoxazole	Nalidixic acid <sup>a</sup>	(other drug)		
	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	
	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	
	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	
	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	S / I / R mm	
<i>E. coli</i> ATCC 25922 (NCCLS QC strain) QC in range? →	Yes No mm	Yes No mm	Yes No mm	Yes No mm	Yes No mm	Yes No mm	

<sup>a</sup> If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

Reviewed by: \_\_\_\_\_ Date of Report: \_\_\_\_\_ / \_\_\_\_\_ / \_\_\_\_\_

- Immunization with available typhoid fever vaccines should be considered only for high-risk populations where epidemic or high endemic rates of multi-drug resistant *S. Typhi* infections are a major cause of morbidity and mortality, **and** where vaccine effectiveness can be formally evaluated.

Consideration of such factors when making decisions based on data will help public health officials meet needs in a manner appropriate to the local situation and antimicrobial susceptibility profile.

## *Shigella*

### IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

The genus *Shigella* is divided into four subgroups: *Shigella dysenteriae* (Subgroup A), *Shigella flexneri* (Subgroup B), *Shigella boydii* (Subgroup C), and *Shigella sonnei* (Subgroup D). Each of these subgroups, with the exception of *S. sonnei*, has several serotypes (Table 15). In general, *S. sonnei* is more common in developed countries and *S. flexneri* and *S. dysenteriae* serotype 1 occur more frequently in developing countries. The proportions of each subgroup varies from country to country, though epidemic dysentery in developing countries is usually caused by *S. dysenteriae* 1, an unusually virulent pathogen. The hallmark of infection with *Shigella* is diarrhea with blood, often termed “dysentery.” However, in almost half of cases, *Shigella* causes acute non-bloody diarrheas that cannot be distinguished clinically from diarrhea caused by other enteric pathogens. Severity of symptoms appears to be dose-related.

*Shigella dysenteriae* serotype 1 differs from other *Shigella* in several ways:

- Only *S. dysenteriae* 1 causes large and prolonged epidemics of dysentery.
- Infection with *S. dysenteriae* 1 causes more severe, more prolonged, and more frequently fatal illness than does infection with other *Shigella*.
- Antimicrobial resistance develops more quickly and occurs more frequently in *S. dysenteriae* 1 than in other *Shigella* groups.

This section of the laboratory manual focuses on the isolation, identification, and antimicrobial susceptibility testing of *Shigella*.

### Identification of *Shigella*

Methods for the collection and transport of fecal specimens and the primary isolation and presumptive identification on selective agar are included in

Appendices 9 and 10. Suspect *Shigella* isolates should be subcultured to a nonselective medium (e.g., Kligler iron agar [KIA] or triple sugar iron agar [TSI]) in preparation for identification by slide serology and biochemical tests. Figure 36 presents a flowchart for the isolation and identification of an isolate as *Shigella*, and Figure 37 provides a sample worksheet which can be used to record test results.

## Biochemical screening tests

Identification of *Shigella* subgroups involves both biochemical and serologic testing. The use of biochemical screening media is usually advisable to avoid wasting antisera. For most laboratories, KIA or TSI will be the single most helpful medium for screening suspected *Shigella* isolates. If an additional test is desired, motility medium can be used to screen isolates before serologic testing is performed.

### *Kligler iron agar and triple sugar iron agar*

To obtain true reactions in KIA, TSI, or other biochemical tests, a pure culture must be used to inoculate the medium. Carefully select at least one of each type of well-isolated colony on each type of plate that was streaked for isolation (i.e., if suspect lactose-nonfermenting colonies that differ in macroscopic appearance are present, a separate test should be run for each.) Using an inoculating needle, lightly touch only the center of the colony. **Do not take the whole colony or go through the colony and touch the surface of the plate** because doing so can pick up contaminants that may be on the surface of the agar. If there is doubt that a particular colony is sufficiently isolated from surrounding colonies, purify the suspicious colony by streaking on another agar plate; afterwards, the KIA slant or TSI slant may be inoculated. Only one colony should be inoculated into each test medium.

KIA and TSI tubes are inoculated by stabbing the butt and streaking the surface of the slant. After incubation for 18–24 hours at 35°–37°C, the slants are observed for reactions typical of *Shigella*. When incubating most biochemicals, caps should be loosened before placement in the incubator. This is particularly important when using KIA and TSI. **If the caps are too tight and anaerobic conditions exist in KIA or TSI, the characteristic reactions of *Shigella* may not occur and a misleading result could be exhibited.** In addition, the KIA and TSI tubes must be prepared so that the tubes have a deep butt (i.e., approximately 3.5 cm) and a long slant (i.e., approximately 2.5 cm). *Shigella* characteristically produces an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H<sub>2</sub>S (see Table 15 and Figure 38). A few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI.

FIGURE 36: Flowchart for the isolation and identification of *Shigella*

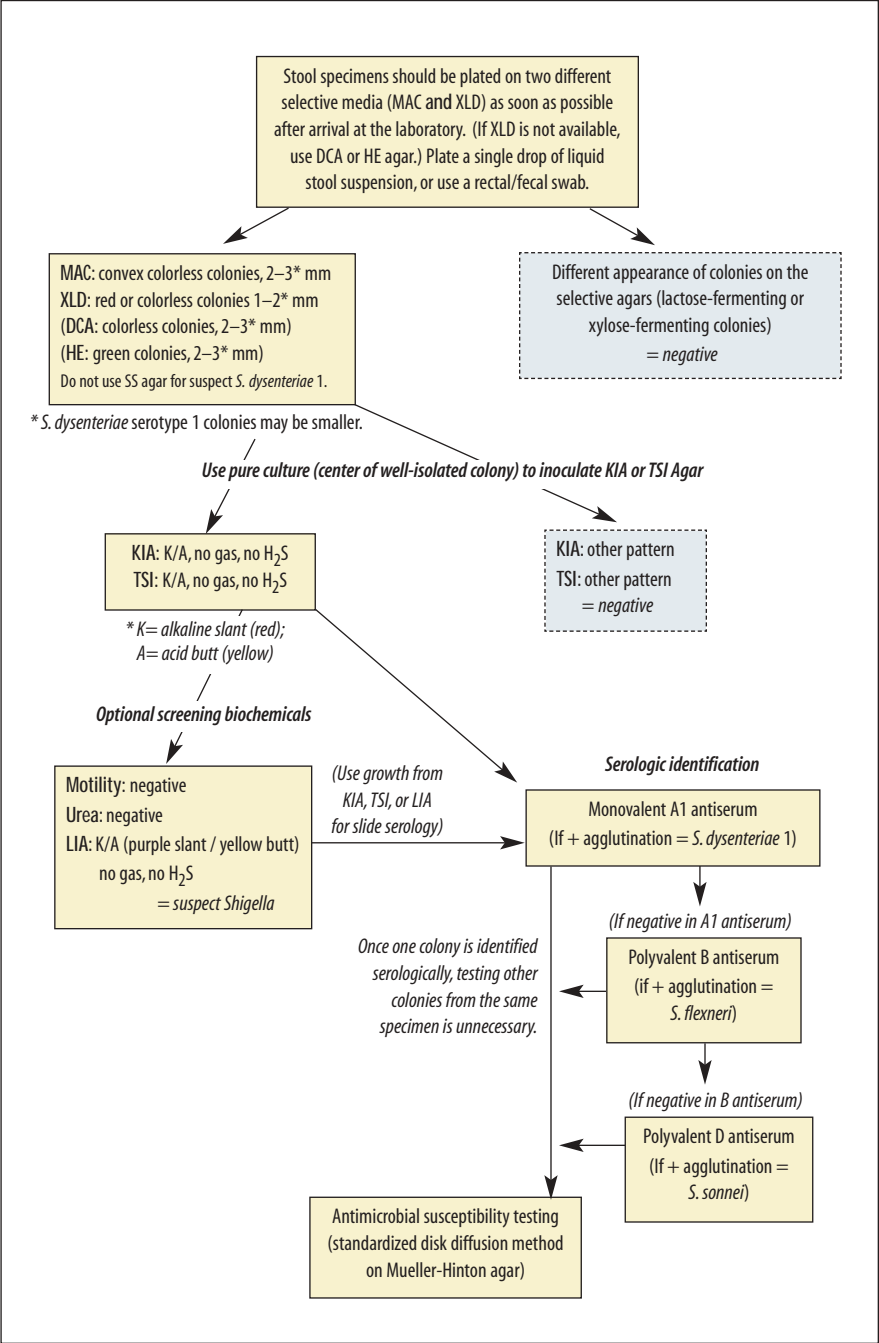


FIGURE 37: Sample worksheet for *Shigella* test results

Specimen number	Medium	XYL/ LAC- <sup>a</sup>	XYL/ LAC+ <sup>a</sup>	Colony <sup>b</sup>	KIA / TSI	OPTIONAL			SLIDE SEROLOGY <sup>c</sup>			Identification
						Motility	Urease	LIA	A1	B	D	
	XLD			X1								
				X2								
				X3								
	MAC			M1								
				M2								
				M3								
	XLD			X1								
				X2								
				X3								
	MAC			M1								
				M2								
				M3								
	XLD			X1								
				X2								
				X3								
	MAC			M1								
				M2								
				M3								

<sup>a</sup> XYL/LAC-: Xylose or Lactose -negative colonies  
XYL/LAC+ : Xylose or Lactose -positive colonies

<sup>b</sup> Identification of only one colony from each suspect case must be confirmed as *Shigella*.

<sup>c</sup> A1 = Monovalent antiserum for *Shigella dysenteriae* (Serogroup A) serotype 1  
B = Polyvalent antiserum for *Shigella flexneri* (Serogroup B)  
D = Polyvalent antiserum for *Shigella sonnei* (Serogroup D)

TABLE 15: Reactions of *Shigella* in screening biochemicals

Screening medium	<i>Shigella</i> reaction	Figure number
Kligler iron agar (KIA)	K/A, no gas produced (red slant/yellow butt) <sup>a</sup>	Figure 38
Triple sugar iron agar (TSI)	K/A, no gas produced (red slant/yellow butt) <sup>a</sup>	~
H <sub>2</sub> S (on KIA or TSI)	Negative ( <i>positive reaction would be blackened medium</i> )	~
Motility	Negative	Figure 39
Urea	Negative	Figure 40
Indole	Positive or Negative	~
Lysine iron agar (LIA)	K/A (purple slant/yellow butt) <sup>b</sup>	Figure 41

<sup>a</sup> K = alkaline (red); A = acid (yellow). Some strains of *S. flexneri* serotype 6 and *S. boydii* produce gas from glucose.

<sup>b</sup> K = alkaline (purple); A = acid (yellow). An alkaline reaction in the butt of the medium indicates that lysine was decarboxylated; an acid reaction in the butt indicates that lysine was not decarboxylated.

**Motility agar**

Motility agar should be inoculated with a straight inoculating needle, making a single stab about 1–2 cm down into the medium. The surface of the motility agar should be dry when used: moisture can cause a non-motile organism to grow down the sides of the agar, creating a haze of growth and appearing to be motile. Motility agar may be inoculated with growth from a KIA or TSI that shows a reaction typical of *Shigella*. Alternatively, motility agar can be inoculated at the same time as the KIA or TSI slant by using the same inoculating needle without touching the colony again. (When motility agar is to be inoculated at the same time as KIA or TSI, use the same colony to first inoculate the motility agar and then to inoculate the KIA or TSI by stabbing the butt and then streaking the surface of the slant. **Do not select a second colony to inoculate the KIA or TSI after the motility agar has been inoculated because it may represent a different organism.**

Examine after overnight incubation at 35°–37°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39). Non-motile organisms do not grow out from the line of inoculation. Motility reactions may be difficult for inexperienced laboratorians to read; therefore, reactions should be compared with positive and negative control strains. *Shigella* are always non-motile (Table 15).

Sulfide-indole-motility medium is a combination medium that is commercially available in dehydrated form (see Appendix 2, “Media, Reagents, and Quality Control”). It can be used in place of motility medium.

**Additional biochemical screening tests**

Other biochemical tests (e.g., urea medium and lysine iron agar [LIA]) may be used for additional screening of isolates before testing with antisera (Table 15).

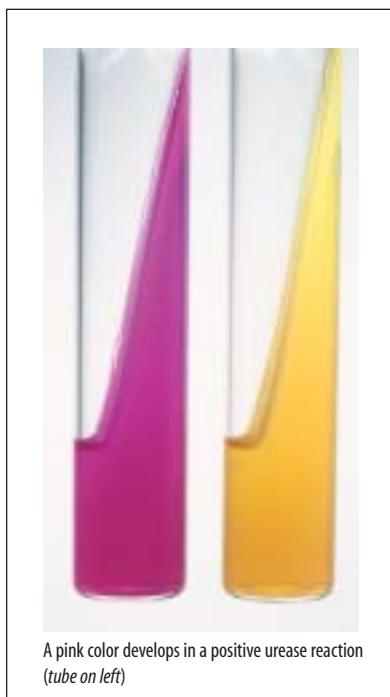
FIGURE 38: Reaction typical of *Shigella* in Kligler iron agar (KIA): alkaline slant and acid butt



FIGURE 39: Motility medium showing a non-motile organism in the left tube and a motile organism (seen as clouding) in the right tube.



FIGURE 40: Reactions in urea medium



The value of these other tests should be assessed before they are used routinely; rationale for performing each test is included along with the following methods. These media, their preparation, and suggested quality control strains are described in Appendix 2.

### *Urea medium*

Urea medium screens out urease-producing organisms (e.g., *Klebsiella* and *Proteus*). Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35°–37°C. **Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color** (Figure 40). Urease-negative organisms do not change the color of the medium, which is a pale yellowish-pink. *Shigella* are always urease-negative.

### *Lysine iron agar*

LIA is helpful for screening out *Hafnia* spp. and certain *E. coli*, *Proteus*, and *Providencia* strains. LIA should be inoculated by stabbing the butt and streaking the slant. After incubation for 18–24 hours at 35°–37°C, organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 41). A blackening of the medium

indicates H<sub>2</sub>S production. Organisms lacking lysine decarboxylase produce an alkaline slant (purple) and an acid butt (yellow), no gas, and no H<sub>2</sub>S. *Proteus* and *Providencia* species will often produce a red slant caused by deamination of the lysine. Lysine iron agar must be prepared so that the tubes have a deep butt (*i.e.*, approximately 3.5 cm). *Shigella* are lysine-negative and characteristically produce an alkaline (purple) slant, an acid (yellow) butt, no gas, and no H<sub>2</sub>S in LIA.

### Serologic identification of *Shigella*

Serologic testing is needed for the identification of *Shigella* isolates. Serologic identification of *Shigella* is performed typically by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype, which is the most frequent cause of severe epidemic dysentery (Table 16). Once one colony from a plate has been identified as *Shigella*, no further colonies from the same specimen need to be tested.

**Laboratorians should be aware that some *Shigella* commercial antiserum is labeled or packaged differently; that is, two packages with different names may contain the same antisera.** For example, *Shigella* polyvalent A, which includes antisera to serotypes 1 through 7, may also be labeled polyvalent A1. Further, monovalent antiserum may be labeled such that it could be confused with polyvalent antiserum; for example, monovalent antiserum to *S. dysenteriae* 1 may be labeled “*Shigella* A1” instead of “*S. dysenteriae* serotype 1”. (Table 16 can serve as a useful guide for referencing which subgroups and serotypes are associated with what *Shigella* nomenclature designation.) When using newly purchased antisera, the laboratorian should read the package insert or check with the manufacturer if the label is not self-explanatory. All lots of antisera should undergo quality control testing before use (Appendix 2).

### Slide agglutination

Because *S. dysenteriae* 1 is the most common agent of epidemic dysentery (followed by *S. flexneri* and *S. sonnei*), isolates that react typically in the screening biochemicals should be screened first with monovalent A1 antiserum, then with polyvalent B antiserum, and finally in polyvalent D antiserum. When a positive agglutination reaction is obtained in one of the antisera, the *Shigella* subgroup is identified, and no further testing with antisera needs to be conducted. (Because subgroup C, *S. boydii*, is so rare it is not cost-effective to perform routine screens for it.)

- a) Agglutination tests may be performed in a Petri dish or on a clean glass slide. Divide the slide into test sections with a wax pencil and place one small drop of physiological saline in each test section on the slide.

FIGURE 41: Reactions in lysine iron agar (LIA) medium



Organisms positive for lysine decarboxylase produce a purple color throughout the LIA medium (*tube on the right*). Lysine-negative organisms produce a yellow color (acid) in the butt portion of the tube (*tube on left*).

TABLE 16: Subgroup and serotype designations of *Shigella*

<i>Shigella</i> (common name)	Subgroup designation (polyvalent antisera)	Serotypes (monovalent antisera)	(Label on commercial antiserum may also say)
<i>S. dysenteriae</i>	Group A	1 – 15 <sup>a,b</sup>	(A1, A2, A3, . . . , A13)
<i>S. flexneri</i>	Group B	1 – 6, X, Y	(B1, B2, B3, B4, B5, B6)
<i>S. boydii</i> <sup>c</sup>	Group C	1 – 19 <sup>b</sup>	(C1, C2, C3, . . . , C18)
<i>S. sonnei</i>	Group D	1	(D1)

<sup>a</sup> Detection of *S. dysenteriae* 1 is of particular importance since it is unusually virulent and causes endemic or epidemic dysentery with high death rates. Monovalent antiserum (absorbed) is required to identify *S. dysenteriae* 1.

<sup>b</sup> Additional provisional serotypes have been reported, but antisera to these new serotypes were not commercially available at the time this manual was printed.

<sup>c</sup> Because *S. boydii* is so rare it is not cost-effective to perform routine screens for it.

- b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, heart-infusion agar (HIA), or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.
- c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 µl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.
- d) Mix the suspension and antiserum well and then tilt the slide back and forth to observe for autoagglutination (Figure 2). The agglutination is more visible if the slide is observed under a bright light and over a black background. If the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping resulting from autoagglutination. If autoagglutination occurs, the culture is termed “rough” and cannot be serotyped.

Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Shigella*.

## Antimicrobial susceptibility testing of *Shigella*

As antimicrobial resistance increases in many parts of the world, monitoring the antimicrobial susceptibility of *Shigella* becomes increasingly important. The disk

FIGURE 42: Serologic identification: agglutination reactions of *Shigella*



diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),<sup>32</sup> and if performed precisely according to the protocol below, will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. However, **any deviation from the method may invalidate the antimicrobial susceptibility test results.** For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing.

Specific methods for determination of antimicrobial susceptibility of *Shigella* are presented in this chapter; however, there are some general guidelines that must first be considered before proceeding: test isolates from the beginning of an outbreak; test appropriate antimicrobial agents; provide timely feedback to public health officials; and, periodically monitor the epidemic for shifts in antimicrobial susceptibility patterns.

<sup>32</sup> Formerly known as the “National Committee for Clinical Laboratory Standards,” NCCLS is now known solely by its acronym.

- *Test the isolates from the beginning of an outbreak*  
**Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic.** That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. (World Health Organization [WHO] surveillance manuals can be useful guides for survey design.)
- *Test appropriate antimicrobial agents*  
**The laboratory should routinely test only those antimicrobial agents that are available in the country or antimicrobial agents that are recommended by WHO as efficacious in the treatment of shigellosis** (Table 17). In addition, if all isolates are resistant to a particular antimicrobial agent (e.g., to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.
- *Provide timely feedback to public health officials*  
**Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials.** The data can then be used to make rational choices for antimicrobial treatment policy.
- *Monitor for changes in antimicrobial susceptibility*  
**As a dysentery epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic.** These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

### Antimicrobial agents for treatment and testing of *Shigella*

The following antimicrobial agents are recommended by the WHO for treatment of *Shigella* infections: ampicillin, ciprofloxacin, norfloxacin, enoxacin, nalidixic acid, pivmecillinam, and trimethoprim-sulfamethoxazole (often referred to as cotrimoxazole).

Antimicrobial agents suggested for use in susceptibility testing of *Shigella* are listed in Table 17; these WHO recommendations are current as of the date of publication of this document.

Testing *Shigella* against certain drugs may yield misleading results when *in vitro* results do not correlate with *in vivo* activity. *Shigella* isolates, for instance, are usually susceptible to aminoglycosides (e.g., gentamicin, kanamycin) and first- and second-generation cephalosporins in the disk diffusion test, but treatment with these drugs is often not effective [NCCLS 2002].

The selection of antimicrobial treatment should be based on the results of recent antimicrobial susceptibility testing of *Shigella* strains obtained from the same region (or from nearby areas if *Shigella* is new to the area). Unfortunately, resistance of *Shigella* to ampicillin and trimethoprim-sulfamethoxazole has become widespread. Nalidixic acid, formerly used as a “backup” drug to treat resistant shigellosis, is now the drug of choice in most areas, but resistance to it has appeared in many places. When resistant to nalidixic acid, *Shigella* should be tested with ciprofloxacin; strains resistant to nalidixic acid often exhibit reduced susceptibility to ciprofloxacin. Pivmecillinam (i.e., amdinocillin pivoxil) is still effective for most strains of *Shigella* but may not be readily available. Fluoroquinolones (e.g., ciprofloxacin, norfloxacin, and enoxacin) are often costly and may not be readily available; fluoroquinolones should be considered only if *Shigella* isolates are resistant to nalidixic acid.

As of the publication of this document (2002), *Shigella* strains are often resistant to ampicillin, trimethoprim-sulfamethoxazole, metronidazole, streptomycin, tetracycline, chloramphenicol, and sulfonamides. In addition, although *Shigella* may be susceptible to some antimicrobial agents *in vitro*, the drug may have no documented efficacy *in vivo*. Examples of such agents are nitrofurans (e.g., nitrofurantoin, furazolidone), aminoglycosides (e.g., gentamicin, kanamycin), first- and second-generation cephalosporins (e.g., cephalexin, cefamandol), and amoxicillin.

TABLE 17: Antimicrobial agents suggested for use in antimicrobial susceptibility testing of *Shigella*

Antimicrobial agents for susceptibility testing of <i>Shigella</i>
Trimethoprim-sulfamethoxazole (cotrimoxazole)
Chloramphenicol
Ampicillin
Nalidixic acid *
<i>*If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.</i>

## Procedure for agar disk diffusion antimicrobial susceptibility testing of *Shigella*

The disk diffusion method of antimicrobial susceptibility testing is similar to that described in the *S. Typhi* chapter, and is summarized in Figure 33. Laboratory diagnostic supplies required for *Shigella* disk diffusion testing are listed in Appendix 9. This section provides seven steps for antimicrobial susceptibility testing of *Shigella* by the disk diffusion method.

### 1. *Mueller-Hinton antimicrobial susceptibility test agar*

Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. **Mueller-Hinton agar, poured to a uniform depth of 3–4mm, should always be used for disk diffusion antimicrobial susceptibility testing**, according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2.

### 2. *McFarland turbidity standard*

A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

### 3. *Preparation of inoculum*

Each culture to be tested should be streaked onto a non-inhibitory agar medium (e.g., blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (e.g., Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. **The bacterial suspension should then be compared to the 0.5 McFarland turbidity standard.** This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (Figures 51 and 52 in the McFarland turbidity standard section of Appendix 2). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth, or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35°C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

#### 4. *Inoculation procedure*

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

#### 5. *Antimicrobial disks*

The working supply of antimicrobial disks should be stored in the refrigerator (at 4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate; this reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before using.

Apply the antimicrobial disks to the plates as soon as possible after the plate is dry, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps or with a mechanical dispensing apparatus, equidistant from each other, and then gently press down onto the agar. In general, **no more than 12 disks are placed on a 150-mm plate and no more than four disks are placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved**. After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours.

#### 6. *Recording and interpreting results*

After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 43) and record it in millimeters. (A sample worksheet is provided in Figure 44.) The measurements can be made with calipers or a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (Table 18), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 44). If there is

both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

- a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)
- b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).
- c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

7. **Quality control**

To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing *Enterobacteriaceae* [e.g., *Shigella*, *Salmonella*, *Escherichia*, *Klebsiella*] and *V. cholerae*.) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 18 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

TABLE 18: Zone size interpretive standards for *Enterobacteriaceae* for selected antimicrobial disks appropriate for the testing of *Shigella*

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint(μg/ml)			NCCLS QC strain <i>E. coli</i> ATCC 25922
		Susceptible	Intermediate	Resistant	
Ampicillin	10 μg	≥ 17 mm (≤ 8 μg/ml)	14 – 16 mm (16 μg/ml)	≤ 13 mm (≥ 32 μg/ml)	16 – 22 mm (2–8 μg/ml)
Chloramphenicol	30 μg	≥ 18 mm (≤ 8 μg/ml)	13 – 17 mm (16 μg/ml)	≤ 12 mm (≥ 32 μg/ml)	21 – 27 mm (2–8 μg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 μg	≥ 16 mm (≤ 2/38 μg/ml)	11 – 15 mm (4/76 μg/ml)	≤ 10 mm (≥ 8/152 μg/ml)	23 – 29 mm (≤ 0.5/9.5 μg/ml)
Nalidixic acid	30 μg	≥ 19 mm (≤ 8 μg/ml)	14 – 18 mm (16 μg/ml)	≤ 13 mm (≥ 32 μg/ml)	22 – 28 mm (1–4 μg/ml)
Ciprofloxacin	5 μg	≥ 21 mm (≤ 1 μg/ml)	16 – 20 mm (2 μg/ml)	≤ 15 mm (≥ 4 μg/ml)	30 – 40 mm (0.004–0.016 μg/ml)

Source: NCCLS (2002) *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, PA 19087 USA.

FIGURE 43: Sample results of the disk diffusion assay



This *Shigella* isolate is resistant to trimethoprim-sulfamethoxazole (cotrimoxazole) and is growing up to the disk (SXT), the zone of which is recorded as 6 mm. In addition to the zones of inhibited growth, note how evenly distributed the growth is on the plate.

**Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

**If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light.

FIGURE 44: Sample form for recording antimicrobial susceptibility test results for *Shigella* isolates

Date of Testing: \_\_\_\_\_ / \_\_\_\_\_ / \_\_\_\_\_  
Test performed by: \_\_\_\_\_

Interpretation of susceptibility: S = susceptible I = intermediate R = resistant

Specimen number	Ampicillin	Chloramphenicol	Trimethoprim-sulfamethoxazole	Nalidixic acid <sup>a</sup>	(other drug)
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
E. coli ATCC 25922 (NCCLS QC strain) QC in range? →	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm

<sup>a</sup> If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin and will probably exhibit reduced susceptibility to ciprofloxacin.

Reviewed by: \_\_\_\_\_

Date of Report: \_\_\_\_\_ / \_\_\_\_\_ / \_\_\_\_\_

**Note:** After 16–18 hours incubation, check the results for the NCCLS–recommended quality control (QC) strain *E. coli* ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 18.)

Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even when the isolates being tested are susceptible.

**If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease;** this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

### **Data for decision-making: *informed epidemic response***

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of the *Shigella* isolates, the information should be reported to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be effective against at least 80% of local *Shigella* strains.
- The antimicrobial agent chosen should be able to be given by mouth.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and antimicrobial susceptibility profile.



## *Vibrio cholerae*

### IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Most *Vibrio cholerae* isolated during cholera outbreaks will be toxigenic serogroup O1 or O139. Because the cultural and biochemical characteristics of these two serogroups are identical, the isolation and identification methods described below apply to both O1 and O139. Both serogroups must be identified using O-group-specific antisera.

Isolates of *V. cholerae* serogroup O1 are classified into two biotypes, El Tor and classical, on the basis of several phenotypic characteristics. Currently, the El Tor biotype is responsible for virtually all of the cholera cases throughout the world, and classical isolates are not encountered outside of India or Bangladesh. In addition, *V. cholerae* O1 is classified into two serotypes (Inaba and Ogawa) on the basis of agglutination in antiserum. A possible third serotype, Hikojima, has been described, but it occurs only rarely. During an outbreak or epidemic, it is worth documenting the biotype and serotype of the isolate; however, it is not necessary to know this information to respond appropriately to the epidemic.

*V. cholerae* serogroup O139 appeared in India in late 1992. It quickly spread to Bangladesh and other Asian countries, although the rate of spread has slowed after the initial outbreaks. Through 1998, 11 countries have officially reported transmission of *V. cholerae* O139 to the World Health Organization (WHO). Imported cases have been reported from the United States and other countries. At this time, endemic *V. cholerae* O139 appears to be confined to Asia.

Fluid replacement is the cornerstone of cholera treatment, and rehydration therapy is a necessity. Antimicrobial therapy is helpful, although not essential, in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and the duration of shedding of vibrios in the feces. When antimicrobial agents are used, it is essential to choose one to which the organism is susceptible. Antimicrobial agents recommended by WHO for treating cholera patients as of the date of publication of this document include tetracycline, doxycycline,

furazolidone, trimethoprim-sulfamethoxazole, erythromycin, or chloramphenicol. Ciprofloxacin and norfloxacin are also effective. Because antimicrobial resistance has been a growing problem in many parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically. Methods for antimicrobial susceptibility testing of *V. cholerae* are addressed in this chapter of the manual, after identification. Isolation and presumptive identification of *V. cholerae* from fecal specimens are included in Appendix 10.

Public health authorities in regions that experience outbreaks of cholera may find that the manual *Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera* [CDC 1999] provides additional helpful discussions about cholera epidemiology and laboratory decision-making in resource-limited regions. The document is available from WHO in English and French; details for ordering are included in Appendix 15.

## Identification of *V. cholerae*

Methods for the collection and transport of fecal specimens and the primary isolation and presumptive identification on selective agar are included in Appendices 9 and 10. Suspect *V. cholerae* isolates should be subcultured to a non-selective medium (e.g., heart infusion agar [HIA] or tryptone soy agar [TSA]) in preparation for identification by slide serology and biochemical tests. *V. cholerae* requires 0.5% NaCl (i.e., 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*. In general, screening with biochemical tests prior to testing with O1 and O139 antisera is not necessary for suspected *V. cholerae* isolates from fecal specimens. However, **if the supply of O-antigen antisera is limited, biochemical tests may be useful for additional screening of isolates before testing them with antisera.** Screening tests and slide serology **must be** performed with growth from **nonselective** media. Figure 45 presents a flowchart for isolation and identification of an isolate as *V. cholerae*, and Figure 46 provides a sample worksheet that can be used to record screening test results.

### Oxidase test

The oxidase test uses Kovac's reagent (a 1% [wt/vol] solution of *N, N, N', N'*-tetramethyl-*p*-phenylenediamine dihydrochloride) to detect the presence of cytochrome *c* in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test can be performed on filter paper or on a swab.

FIGURE 45: Flowchart for isolation and identification of *Vibrio cholerae*

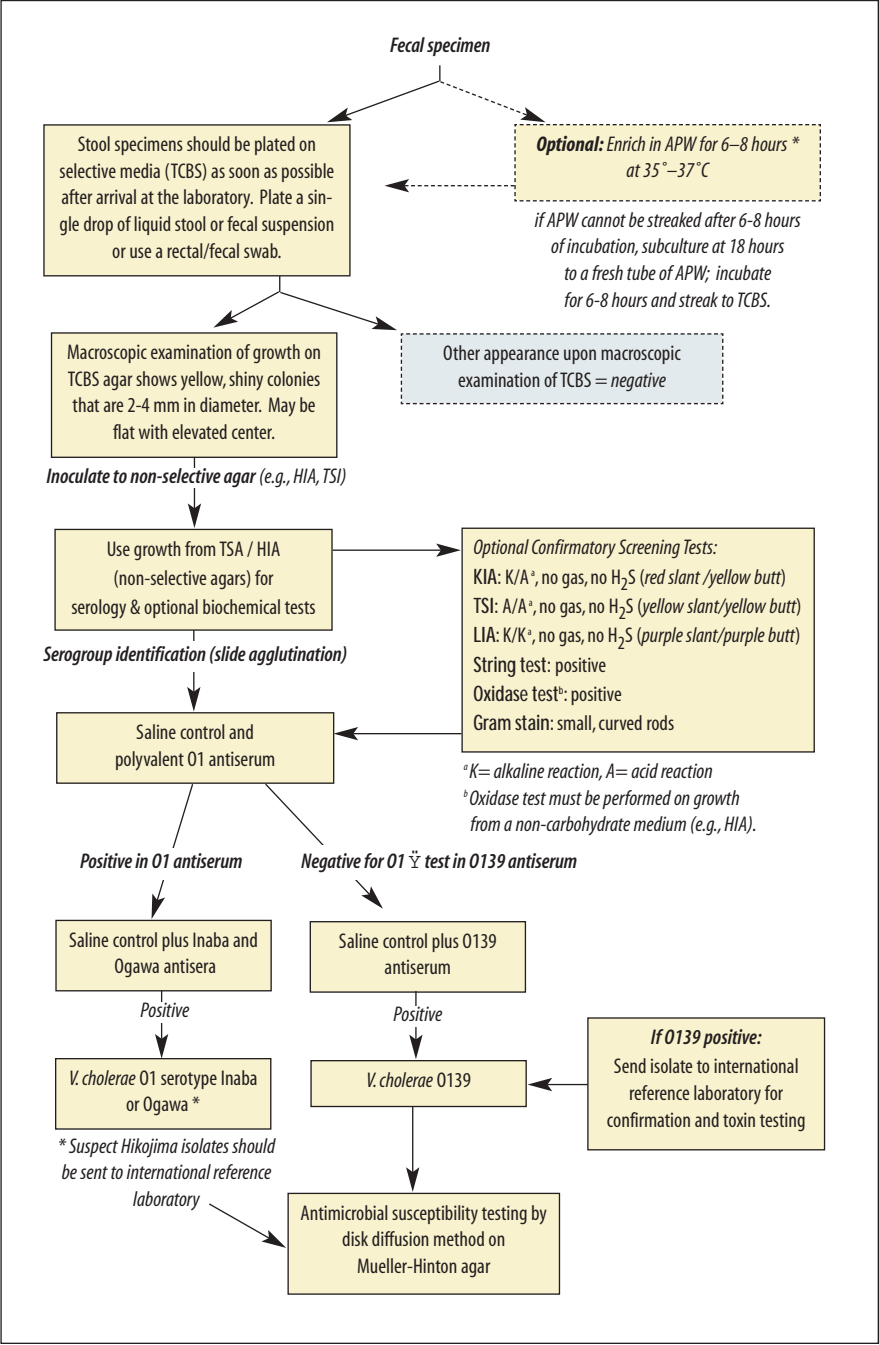


FIGURE 46. Sample worksheet for *Vibrio cholerae* test results

Specimen number	Medium	SUC <sup>+b</sup>	SUC <sup>-c</sup>	Colony	OPTIONAL			SLIDE SEROLOGY <sup>d</sup>				Identification	
					Oxidase test	String test	Gram stain or wet mount	PV 01	Inaba	Ogawa	0139		
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS <sup>a</sup>			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS <sup>a</sup>			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS <sup>a</sup>			AT1									
				AT2									
				AT3									

It is only necessary to identify one colony **from each suspect case as *V. cholerae***

<sup>a</sup> APW-TCBS :alkaline peptone water enrichment prior to inoculating TCBS

<sup>b</sup> SUC + : Sucrose-positive colonies

<sup>c</sup> SUC - : Sucrose-negative colonies

<sup>d</sup> PV01 = polyvalent antiserum for *V. cholerae* serogroup 01  
Inaba = monovalent antiserum for *V. cholerae* 01 serotype Inaba  
Ogawa = monovalent antiserum for *V. cholerae* 01 serotype Ogawa  
0139 = monovalent antiserum for *V. cholerae* serogroup 0139

Perform the oxidase test with fresh growth from an HIA or TSA slant or any non-selective, non-carbohydrate-containing medium; **do not use growth from thiosulfate citrate bile-salts sucrose [TCBS] agar** because it may yield either false-negative or false-positive results. **Do not perform this test with a Nichrome loop**, as it may produce a false-positive reaction. Positive and negative controls should be tested at the same time as the test isolate for quality control purposes. Preparation of the oxidase reagent is described in Appendix 2.

### ***Moistened filter paper method***

- a) Add two to three drops of Kovac's oxidase reagent to a piece of filter paper in a petri dish and allow it to absorb; the filter paper should be moist (but not wet) after the reagent has been absorbed.
- b) Using a platinum loop, a plastic loop, a sterile swab, or a sterile wooden applicator stick or toothpick, pick a portion of the colony to be tested **from non-selective media** and rub it onto the moistened filter paper. (**Do not use a Nichrome loop.**)
- c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur in the region where the growth has been smeared within 10 seconds (Figure 10).

### ***Swab method***

- a) Pick up suspect colonies from a non-selective culture plate or growth from a non-selective agar slant with the swab.
- b) Use a Pasteur pipette to add one drop of Kovac's oxidase reagent to the swab.
- c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur within 10 seconds. (See Figure 20).

If an isolate has not turned purple within 10 seconds of adding the Kovac's oxidase reagent, **it is not considered oxidase-positive**. Organisms of the genera *Vibrio* (Table 19), *Neisseria*, *Campylobacter*, *Aeromonas*, *Plesiomonas*, *Pseudomonas*, and *Alcaligenes* are all oxidase-positive; all *Enterobacteriaceae* are oxidase-negative.

### **Additional biochemical screening tests**

The string reaction, Kligler iron agar (KIA) or triple sugar iron agar (TSI), lysine iron agar (LIA), Gram stain, and wet mount for motility are other possible tests that may be used for additional screening of isolates before testing with antisera (Table 19). The value of these other tests should be assessed before they are used routinely; rationale for performing each test (e.g., use of the string test to rule out *Aeromonas*) is included along with the following methods. These media, their preparation, and suggested quality control strains are described in Appendix 2.

TABLE 19: Reactions of *Vibrio cholerae* in screening tests

Screening test	<i>Vibrio cholerae</i> reactions	Figure number
Oxidase test	Positive	Figure 10 and Figure 20
String test	Positive	Figure 47
Kligler iron agar (KIA)	K/A (red slant/yellow butt) <sup>a</sup> , no gas produced, no H <sub>2</sub> S [18–24 hours]	Figure 48
Triple sugar iron agar (TSI)	A/A (yellow slant/yellow butt) <sup>a</sup> , no gas produced, no H <sub>2</sub> S [18–24 hours]	Figure 48
Lysine iron agar (LIA)	K/K (purple slant / purple butt) <sup>a,b</sup> , no gas produced, no H <sub>2</sub> S [18–24 hours]	–
Gram stain	Small, gram-negative curved rods	–
Wet mount	Small, curved rods with darting motility	–

<sup>a</sup>K= alkaline; A= acid

<sup>b</sup> An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt indicates that lysine was not decarboxylated.

### String test

The string test uses fresh growth from nonselective agar and is useful for ruling out non-*Vibrio* species, particularly *Aeromonas* species. The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from heart infusion agar (or other noninhibitory medium) in a drop of 0.5% aqueous solution of sodium deoxycholate. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells, causing the mixture to become viscous. A mucoid “string” is formed when an inoculating loop is drawn slowly away from the suspension (Figure 47). *V. cholerae* strains are positive, whereas *Aeromonas* strains are usually negative (Table 19). Other *Vibrio* species may give a positive or weak string test reaction.

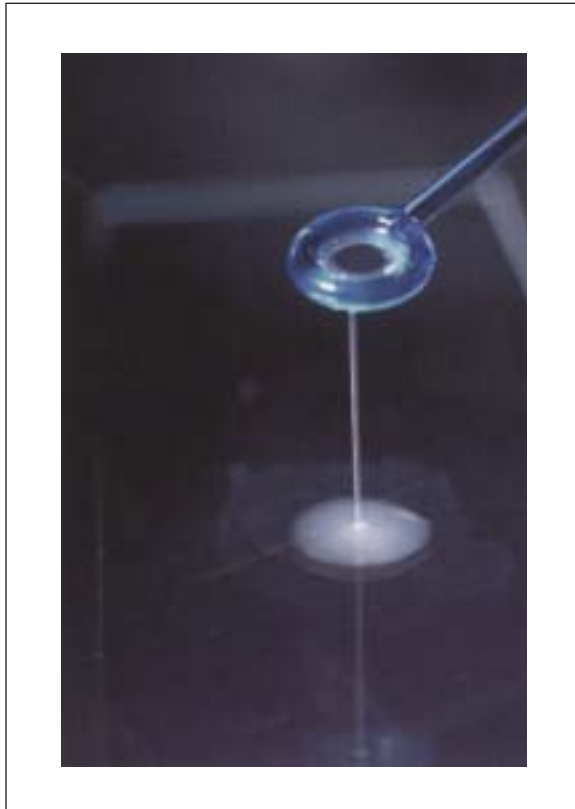
### Kligler iron agar and triple sugar iron agar

KIA and TSI can be used to rule out *Pseudomonas* species and certain *Enterobacteriaceae*. **It is important that Kligler iron agar and triple sugar iron agar be prepared so the tubes have a deep butt and a long slant;** if the butt is not deep enough, misleading reactions may occur in these media (Appendix 2). A tube prepared so that the butt is approximately 3.5-cm deep and the slant is approximately 2.5-cm is acceptable.

KIA or TSI agar slants are inoculated by stabbing the butt and streaking the surface of the medium. Incubate the slants at 35°–37°C and examine after 18–24 hours.

**Caps on all tubes of biochemical media should be loosened before incubation, but this is particularly important for KIA or TSI slants. If the caps are too tight**

FIGURE 47: A positive string test with *Vibrio cholerae*



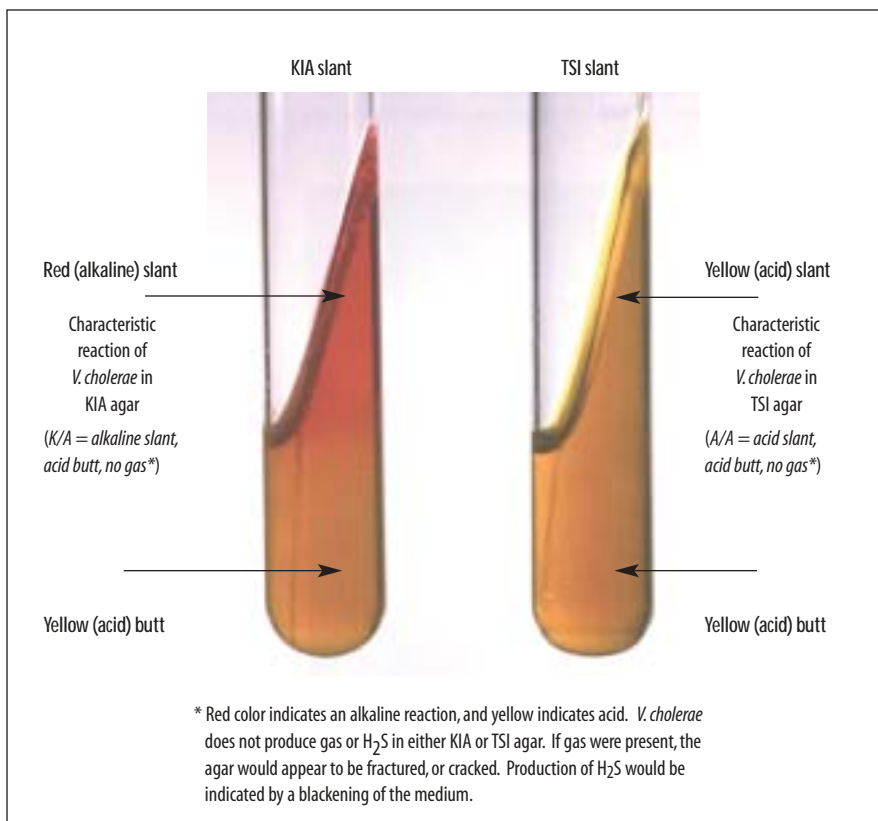
**and anaerobic conditions exist in the tube, an inappropriate reaction will occur** and the characteristic reactions of *V. cholerae* may not be exhibited.

The reactions of *V. cholerae* on KIA, which contains glucose and lactose, are similar to those of lactose-nonfermenting *Enterobacteriaceae* (i.e., alkaline [red] slant, acid [yellow] butt, no gas, and no H<sub>2</sub>S). However, on TSI, *V. cholerae* strains produce an acid (yellow) slant, acid (yellow) butt, no gas, and no H<sub>2</sub>S (Table 19 and Figure 48).

### ***Lysine iron agar***

LIA is helpful for screening out *Aeromonas* and certain *Vibrio* species, which, unlike *V. cholerae*, do not decarboxylate lysine. LIA must be prepared so that the tubes have a deep butt (approximately 3.5 cm) and a long slant (approximately 2.5 cm). As with KIA and TSI, if the butt is not deep enough, misleading reactions may occur in this medium. In LIA, the decarboxylation of lysine occurs only in anaerobic conditions and a false-negative reaction may result from insufficient medium in the tube (Appendix 2). Inoculate LIA by stabbing the butt and then

FIGURE 48: Reactions of *Vibrio cholerae* in Kligler iron agar (KIA) and triple sugar iron agar (TSI)



streaking the slant; after incubation for 18–24 hours at 35°–37°C, examine the LIA slants for reactions typical of *V. cholerae*. Organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the tube (Figure 41); organisms without the enzyme produce an acid reaction (yellow color) in the butt portion of the tube. H<sub>2</sub>S production is indicated by a blackening of the medium. The LIA reaction for *V. cholerae* is typically an alkaline slant (purple), alkaline butt (purple), no gas, and no H<sub>2</sub>S (Table 19). *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine.

### Gram stain

Examining overnight growth of *Vibrio cholerae* from a heart infusion agar slant by Gram stain will demonstrate typical small, curved gram-negative rods (Table 19). Staining with crystal violet only is a more rapid technique that will also demonstrate the cell morphology typical of *Vibrio* species.

### ***Wet mount***

Dark-field and phase-contrast microscopy have been used for screening suspected isolates of *V. cholerae*. With these techniques, saline suspensions are microscopically examined for the presence of organisms with typical small, curved rods and darting (*i.e.*, “shooting star”) motility (Table 19).

### **Serologic identification of *V. cholerae* O1 and O139**

Following presumptive biochemical identification of the agent as *V. cholerae*, it is appropriate to confirm identification with serology. **If an epidemic of cholera is suspected, the most common cause is *V. cholerae* O1. If *V. cholerae* O1 is not isolated, the laboratory should test for *V. cholerae* O139. If neither of these organisms is isolated, arrangements should be made to send stool specimens to a reference laboratory.** Local and regional laboratories should send isolates requiring testing with O139 antiserum to the national reference laboratory; if the national reference laboratory is still unable to confirm the identification of a *V. cholerae* isolate as O1 or O139, an international reference laboratory can provide guidance.

To conserve resources, the laboratory can first test *V. cholerae* for somatic O1 antigens, and then test with O139 antiserum only if the isolate does not yield a positive agglutination reaction in the O1 antiserum.

### ***Presumptive identification using O1 and O139 antisera***

For slide agglutination testing with polyvalent O1 or O139 antisera, fresh growth of suspected *V. cholerae* from a nonselective agar medium should be used. **(Using growth from thiosulfate citrate bile salts sucrose (TCBS) agar may result in false-negative reactions.)** After 5–6 hours of incubation, growth on the surface of the slant is usually sufficient to perform slide agglutination with antisera; if not, incubate for a longer period. If the isolate does not agglutinate in O1 antiserum, test in O139 antiserum. If it is positive in the polyvalent O1 or in the O139 antiserum, the isolate may be reported as presumptive *V. cholerae* O1 or O139. Presumptive *V. cholerae* O1 isolates should be tested in monovalent Ogawa and Inaba antisera (methods follow this section). Once one colony from a plate has been identified as *V. cholerae* O1 or O139, no further colonies from the same plate need to be tested. [Refer to Appendix 2 for a discussion on quality control of antisera.]

### ***Confirmation of *V. cholerae* O1 using Inaba and Ogawa antisera***

The O1 serogroup of *V. cholerae* has been further divided into three serotypes: Inaba, Ogawa, and Hikojima (which is very rare). Serotype identification is based on agglutination in monovalent antisera to type-specific O antigens (Table 20). A positive reaction in either Inaba or Ogawa antiserum is sufficient to confirm the identification of a *V. cholerae* O1 isolate. Isolates that agglutinate weakly or slowly with serogroup O1 antiserum but do not agglutinate with either Inaba or Ogawa

antiserum are not considered to be serogroup O1. Identifying these antigens is valid only with serogroup O1 isolates. For this reason, Inaba and Ogawa antisera should never be used with strains that are negative with polyvalent O1 antiserum.

Strains of one serotype frequently produce slow or weak agglutination in antiserum to the other serotype, depending on how well the serotype-specific antisera have been absorbed. For this reason, **agglutination reactions with Inaba and Ogawa antisera should be examined simultaneously, and the strongest and most rapid reaction should be used to identify the serotype.** With adequately absorbed antisera, strains that agglutinate very strongly and equally with both the Ogawa and Inaba antisera are rarely, if ever, encountered. If such reactions are suspected, the strains should be referred to a reference laboratory for further examination and may be referred to as “possible serotype Hikojima.”

*Slide agglutination procedure*

Agglutination tests for *V. cholerae* somatic O antigens may be conducted in a Petri dish or on a clean glass slide.

- a) Divide the slide into test sections with a wax pencil and place one small drop of physiological saline in each test section on the slide.
- b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, HIA, or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.
- c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 µl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.

TABLE 20: Agglutination reactions in absorbed antiserum of serotypes of *Vibrio cholerae* serogroup O1

<i>V. cholerae</i> O1 serotype	Ogawa antiserum	Inaba antiserum
Ogawa	+ <sup>a</sup>	– <sup>b</sup>
Inaba	–	+
Hikojima <sup>c</sup>	+	+

<sup>a</sup> + indicates a positive agglutination reaction in the absorbed antiserum.

<sup>b</sup> – indicates a negative agglutination reaction in the absorbed antiserum.

<sup>c</sup> If there is a positive reaction in both Ogawa and Inaba antisera and the Hikojima serotype is suspected, send the isolate to an international reference laboratory, following packing regulations as presented in Appendix 12.

- d) Mix the suspension and antiserum well and then tilt the slide back and forth to observe for autoagglutination (Figure 2). The agglutination is more visible if the slide is observed under a bright light and over a black background. If the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping resulting from autoagglutination. If autoagglutination occurs, the culture is termed “rough” and cannot be serotyped.

#### *Confirmation of V. cholerae O139*

A suspected *V. cholerae* isolate that reacts in O139 antiserum but not in polyvalent O1 antiserum should be sent to a reference laboratory. Confirmation of *V. cholerae* O139 includes testing for production of cholera enterotoxin and verification of the O139 antigen by slide agglutination with O139 antiserum. No serotypes have been identified in the O139 serogroup. Enterotoxin assays (e.g., PCR, EIA, DNA probing) are complex and beyond the scope of this manual. Few laboratories are capable of doing these tests, and they are performed mainly by international reference laboratories. (See Appendix 12 for packing and shipping regulations and Appendix 14 for a list of international reference laboratories.)

Following identification of the agent, it is appropriate for the laboratorian to commence testing for antimicrobial susceptibility patterns if antimicrobial agents are to be used to treat the cholera outbreak.

### **Antimicrobial susceptibility testing of *V. cholerae***

As antimicrobial resistance increases in many parts of the world, monitoring the antimicrobial susceptibility of *Vibrio cholerae* O1 and O139 has become increasingly important. The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),<sup>33</sup> and if performed precisely according to the protocol below, will provide data that can reliably predict the in vivo effectiveness of the drug in question. However, **any deviation from the testing method may invalidate the antimicrobial susceptibility test results.** For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing.

Specific methods for determination of antimicrobial susceptibility patterns of *V. cholerae* are presented in this manual; however, there are some general guidelines that must first be considered before proceeding: test isolates from the beginning of

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<sup>33</sup> Formerly known as the “National Committee for Clinical Laboratory Standards,” NCCLS is now known solely by its acronym.

an outbreak; test appropriate antimicrobial agents; provide timely feedback to public health officials; and, periodically monitor the epidemic for shifts in antimicrobial susceptibility patterns.

- *Test the isolates from the beginning of the outbreak*  
**Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic.** That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. WHO surveillance manuals can provide guidance in the development of such surveys.
- *Test appropriate antimicrobial agents*  
**The laboratory should not routinely test antimicrobial agents that are not available in the country or antimicrobial agents that are not recommended by WHO as efficacious in the treatment of cholera** (Table 21). In addition, if all isolates are resistant to a particular antimicrobial agent (e.g., to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory (Appendix 14) can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.
- *Provide timely feedback to public health officials*  
**Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials.** The data can then be used to make rational choices for antimicrobial treatment policy.
- *Monitor for changes in antimicrobial susceptibility*  
**As a cholera epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic.** These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

The antimicrobial agents recommended by the WHO for testing of *V. cholerae* are included in Table 21; these recommendations are current as of 2002.

In addition to the general principles of antimicrobial susceptibility testing presented in the previous section, there are several special considerations to be heeded when performing disk diffusion testing of *Vibrio cholerae*:

TABLE 21: Antimicrobial agents suggested for use in susceptibility testing of *Vibrio cholerae* O1 and O139

Antimicrobial agents for susceptibility testing of <i>V. cholerae</i>	
Trimethoprim-sulfamethoxazole (cotrimoxazole)	
Furazolidone	
Tetracycline <sup>a</sup>	
Nalidixic acid <sup>b</sup>	
<sup>a</sup> The results from the tetracycline disk are also used to predict susceptibility to doxycycline.	
<sup>b</sup> If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.	

- Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, zone size interpretive criteria for *V. cholerae* O1 and O139 have been established by NCCLS only for ampicillin, chloramphenicol, sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole. The interpretations of susceptible, intermediate and resistant for isolates tested against these drugs by disk diffusion correlate well with the minimum inhibitory concentration (MIC) results determined by broth microdilution.
  - Disk diffusion tests should not be used for doxycycline and erythromycin because the results for these drugs are frequently inaccurate for *V. cholerae* O1 and O139 strains. Therefore, these agents should not be tested using this method.
  - The results from the tetracycline disk should be used to predict susceptibility to doxycycline. If a strain is susceptible to tetracycline, it will also be susceptible to doxycycline.
  - At this time there is no *in vitro* method to accurately determine susceptibility to erythromycin.
  - The reliability of disk diffusion results for other antimicrobial agents, including ciprofloxacin, furazolidone, and nalidixic acid, has not been validated.
- 3 Until interpretive criteria have been established for *V. cholerae*, disk diffusion may be used to screen *V. cholerae* for resistance to ciprofloxacin, using NCCLS interpretive criteria for the *Enterobacteriaceae* (Table 22).
- 3 Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* based on multi-laboratory studies using NCCLS testing methodologies. When screening with the disk diffusion method for these agents, results should be interpreted with caution (Table 22).

## Procedure for agar disk diffusion antimicrobial susceptibility testing of *V. cholerae*

Laboratory diagnostic supplies required for *V. cholerae* disk diffusion testing are listed in Appendix 9. Figure 33 summarizes the disk diffusion method of antimicrobial susceptibility testing for enteric bacterial pathogens. The following section provides seven steps for antimicrobial susceptibility testing of *Vibrio cholerae* by the disk diffusion method.

### 1. *Mueller-Hinton antimicrobial susceptibility test agar*

**Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar, poured to a uniform depth of 3–4 mm, should always be used for disk diffusion antimicrobial susceptibility testing,** according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can affect disk diffusion test results, this medium should be prepared strictly according to the methods and quality control instructions presented in Appendix 2.

### 2. *McFarland turbidity standard*

A 0.5 McFarland turbidity standard should be prepared and quality controlled prior to beginning antimicrobial susceptibility testing (Appendix 2, Figure 50). If tightly sealed to prevent evaporation and stored in the dark, the turbidity standard can be stored for up to 6 months. The 0.5 McFarland turbidity standard is used to adjust the turbidity of the inoculum for the antimicrobial susceptibility test.

### 3. *Preparation of inoculum*

Each culture to be tested should be streaked onto a non-inhibitory agar medium (e.g., blood agar, brain heart infusion agar, or tryptone soy agar [TSA]) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or nonselective broth (e.g., Mueller-Hinton broth, heart infusion broth, or tryptone soy broth [TSB]) and vortex thoroughly. **The bacterial suspension should then be compared to the 0.5 McFarland turbidity standard.** This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (Figures 51 and 52 in the McFarland turbidity standard section of Appendix 2). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the 0.5 McFarland turbidity standard, the turbidity can be reduced by adding sterile saline or broth, or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Pick four or five colonies from overnight growth on agar and inoculate them into

broth (Mueller-Hinton broth, heart infusion broth, or TSB). Incubate the broth at 35°C until turbid (usually 16–24 hours), and then adjust the turbidity to the proper density.

#### 4. Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.

If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture), laboratorians may choose to prepare a purity plate to ensure the suspension used for antimicrobial susceptibility testing is pure. To prepare the purity plate, after inoculating the Mueller-Hinton agar plate for confluent growth, label (a portion of) a separate TSA plate (or other non-selective medium) and **use the same swab** of suspension with which the Mueller-Hinton was inoculated to streak for isolation; do not place the swab back into the suspension. Several inocula can be streaked on different sections of a properly labeled purity plate, but the streaks **must not overlap**.

#### 5. Antimicrobial disks

The working supply of antimicrobial disks should be stored in the refrigerator (at 4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use.

Apply the antimicrobial disks to the plates as soon as possible after they are dry, but no longer than 15 minutes after inoculation. Place each disk individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. In general, **no more than 12 disks are placed on a 150-mm plate and no more than four disks are placed on a 100-mm plate** to prevent overlapping of the zones of inhibition and possible resultant error in measurement. Diffusion of the drug in the disk begins immediately; therefore, **once a disk contacts the agar surface, the disk should not be moved**. After the disks are placed on the plate, invert the plate and incubate at 35°C for 16–18 hours; if a purity plate was prepared, incubate it under the same conditions.

## 6. Recording and interpreting results

After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) in millimeters. (Figure 43 shows growth, Figure 6 and Figure 28 show how to measure the zones, and Figure 49 presents a sample worksheet in which to record data.) The measurements can be made with calipers or a ruler on the under-surface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (approximately 80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (Table 22), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. Measure the distance from the inner-most colonies (*i.e.*, those closest to the disk) to the center of the antimicrobial disk, and double this measurement to obtain the diameter; record the measurement and interpretation of antimicrobial susceptibility (Figure 49). If there is both an inner- and outer zone of inhibition of growth around the antimicrobial disk:

- a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)
- b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).
- c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

The presence of colonies within a zone of inhibition may predict eventual resistance to that antimicrobial agent.

## 7. Quality control of agar disk diffusion antimicrobial susceptibility testing

To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test. (ATCC 25922 is the *E. coli* control strain used when testing Enterobacteriaceae [*e.g.*, *Shigella*, *Salmonella*, *Escherichia*, *Klebsiella*] and *V. cholerae*.) Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits; Table 22 includes the diameters of the zones of inhibition for ATCC 25922. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

**Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors.** The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For

FIGURE 49. Sample form for recording antimicrobial susceptibility results for *Vibrio cholerae*

Date of Testing: \_\_\_\_/\_\_\_\_/\_\_\_\_

Test performed by: \_\_\_\_\_

Interpretation of susceptibility: S = susceptible I = intermediate R = resistant

Specimen number	Furazolidone <sup>a</sup>	Trimethoprim-sulfamethoxazole	Tetracycline <sup>b</sup>	Nalidixic acid <sup>a</sup>	(other drug)
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
<i>E. coli</i> ATCC 25922 (NCCLS QC strain)	S I R mm	S I R mm	S I R mm	S I R mm	S I R mm
QC in range? →	Yes No	Yes No	Yes No	Yes No	Yes No

<sup>a</sup> Proposed interpretive criteria based on multi-laboratory studies. (NCCLS criteria have not been established for *V. cholerae* with furazolidone or nalidixic acid.)  
If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

<sup>b</sup> Susceptibility of a *V. cholerae* isolate to tetracycline is used to predict susceptibility to doxycycline as well as tetracycline (but doxycycline is not tested directly).

Reviewed by : \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

*Note:* After 16–18 hours incubation, check the results for the NCCLS-recommended quality control (QC) strain *E. coli* ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 22.)

TABLE 22: Interpretive standards for antimicrobial susceptibility testing of *Vibrio cholerae* with selected antimicrobial disks

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)			(NCCLS QC strain <i>E. coli</i> ATCC 25922)
		Susceptible	Intermediate	Resistant	
Ampicillin <sup>a</sup>	10 µg	≥ 17 mm (≤ 8 µg/ml)	14 – 16 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	16 – 22 mm (2–8 µg/ml)
Chloramphenicol <sup>a,b</sup>	30 µg	≥ 18 mm (≤ 8 µg/ml)	13 – 17 mm (16 µg/ml)	≤ 12 mm (≥ 32 µg/ml)	21 – 27 mm (2–8 µg/ml)
Furazolidone <sup>c</sup>	100 µg	≥ 18 mm	-	< 18 mm	22 – 26 mm <sup>d</sup> -
Nalidixic acid <sup>c</sup>	30 µg	≥ 19 mm	-	< 19 mm	22 – 28 mm (1–4 µg/ml)
Ciprofloxacin <sup>e</sup>	5 µg	≥ 21 mm (≤ 1 µg/ml)	16 – 20 mm (2 µg/ml)	≤ 15 mm (≥ 4 µg/ml)	30 – 40 mm (0.004–0.016 µg/ml)
Tetracycline <sup>a</sup>	30 µg	≥ 19 mm (≤ 4 µg/ml)	15 – 18 mm (8 µg/ml)	≤ 14 mm (> 16 µg/ml)	18 – 25 mm (0.5–2 µg/ml)
Trimethoprim-sulfamethoxazole <sup>a</sup> (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm (≤ 2/38 µg/ml)	11 – 15 mm (4/76 µg/ml)	≤ 10 mm (≥ 8/152 µg/ml)	23 – 29 mm (≤ 0.5/9.5 µg/ml)

<sup>a</sup> Source: NCCLS (2002) *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, PA 19087 USA.

<sup>b</sup> Use these interpretive standards for chloramphenicol with caution because the disk diffusion test may misclassify many organisms (high minor error rate) [NCCLS 2002].

<sup>c</sup> Proposed interpretative criteria based on multi-laboratory studies; criteria have not been established for *V. cholerae* by NCCLS.

<sup>d</sup> Quality control inhibition zone diameter ranges for furazolidone have not been validated by NCCLS; the ranges presented in this table are based on those suggested by the manufacturer of the antimicrobial disks.

<sup>e</sup> Criteria for interpretation of susceptibility of *V. cholerae* to ciprofloxacin have not been developed; this table presents tentative interpretive criteria based on NCCLS interpretive criteria for *Enterobacteriaceae*.

example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

**If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even the isolates being tested are susceptible. Again, as mentioned above, erythromycin tested against *V. cholerae* will give

misleading results because these *in vitro* results do not necessarily correlate with *in vivo* activity.

**If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease;** this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

### **Data for decision-making: *informed epidemic response***

Once the laboratory has assessed the identity and antimicrobial susceptibility patterns of the *V. cholerae* O1 or O139 isolates, the information should be reported back to public health officials in a timely manner. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be effective against at least 80% of local *V. cholerae* O1/O139 strains. Evidence of clinical efficacy is the most important criterion, especially for a drug such as erythromycin, which cannot be tested *in vitro*.
- The antimicrobial agent chosen should be able to be given by mouth.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in manner appropriate to the local situation and antimicrobial susceptibility profile.



## Conclusion

The techniques and media described in this manual adhere to internationally recognized clinical standards. The procedures provide laboratorians from regions with limited resources with the methodological tools needed for the quality-controlled detection of antimicrobial resistance in seven pathogens causing acute bacterial infections of public health importance. Application of these methods will enable laboratorians to make valid comparisons and interpretations of their findings within countries and across borders.

This manual addresses the identification and antimicrobial susceptibility testing of bacterial pathogens that cause acute respiratory infections, meningitis, febrile illness, diarrheal disease, and sexually transmitted infections of public health concern. *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* are three pathogens contributing to a substantial proportion of bacterial pneumonia and meningitis morbidity and mortality; commonly used antimicrobial agents (e.g., penicillin and trimethoprim-sulfamethoxazole) are becoming decreasingly effective for treatment of these pathogens. Laboratory data on antimicrobial susceptibility and serotype (or serogroup) distributions can help determine not only if antibiotic treatment or prophylaxis is appropriate, but also if vaccination would be efficacious. Antimicrobial resistance in *Neisseria gonorrhoeae* is a growing concern not only because of its direct health effects on the reproductive tract, but also because epidemiological evidence indicates that gonorrhea infection facilitates transmission of HIV/AIDS. Typhoid fever, a disease caused by *Salmonella* serotype Typhi, is endemic in many developing countries, and outbreaks of multi-drug resistant strains have been reported worldwide. *Shigella* is frequently the agent of epidemic bloody diarrhea and has become progressively more resistant to commonly available and affordable treatment regimens. Cholera, an internationally reportable disease caused by *Vibrio cholerae* O1 and O139 that is clinically recognized by the presentation of abundant watery diarrhea, must be treated primarily with rehydration therapy, but antimicrobial

agents contribute to the reduction of stool volume. As antimicrobial resistant strains of disease spread throughout communities and more people become infected with less-treatable bacteria, the burden on public health and on social and economic development will continue to grow.

A goal of this manual has been to provide public health reference laboratories with a tool to produce standardized antimicrobial susceptibility test results that can be used for public health decision-making. Individual results of antimicrobial susceptibility tests are important for clinical treatment plans; adequate information must be provided to health-care providers. Laboratorians have the power and responsibility to contribute to the shaping of local policy for prevention, control, and treatment of disease by communicating patterns of a pathogen's antimicrobial susceptibility to public health officials. Concerted public health efforts are needed to reduce the frequency and spectrum of antimicrobial resistant disease in both hospital and community settings.

## Standard Safety Practices in the Microbiology Laboratory

Laboratorians working with infectious agents are subject to laboratory-acquired infections as a result of accidents or unrecognized incidents. The degree of hazard depends upon the virulence of the biological agent concerned and host resistance. Laboratory-acquired infections occur when microorganisms are inadvertently ingested, inhaled, or introduced into the tissues. Laboratorians are relatively safe when working with *Haemophilus influenzae* and *Streptococcus pneumoniae*; however, persons who work with aerosolized *Neisseria meningitidis* are at increased risk of acquiring a meningococcal infection. The primary laboratory hazard associated with enteric pathogens such as *Shigella*, *Vibrio* or *Salmonella* is accidental ingestion. Biosafety Level 2 (BSL-2) practices are suitable for work involving these agents that present a moderate potential hazard to personnel and the environment. The following requirements have been established for laboratorians working in BSL-2 facilities.

- Laboratory personnel must receive specific training in handling pathogenic agents and be directed by competent scientists.
- Access to the laboratory must be limited when work is being conducted.
- Extreme precautions must be taken with contaminated sharp items.
- Certain procedures involving the creation of infectious aerosols or splashes must be conducted by personnel who are wearing protective clothing and equipment.

### Standard microbiological safety practices

The following safety guidelines listed below apply to all microbiology laboratories, regardless of biosafety level.

#### Limiting access to laboratory

Sometimes, people who do not work in the laboratory attempt to enter the laboratory to look for test results they desire. Although this occurs more frequently in clinical laboratories, access to the laboratory should be limited, regardless of the setting.

Biohazard signs or stickers should be posted near all laboratory doors and on all equipment used for laboratory work (*e.g.*, incubators, hoods, refrigerators, and freezers). Children under 12 years of age and pets are not allowed in laboratory areas. All laboratories should be locked when not in use. In addition, all freezers and refrigerators located in corridors should be locked.

## Handwashing

Each laboratory should contain a sink for handwashing. Hands should be washed for at least one minute. Frequent handwashing is one of the most effective procedures for avoiding laboratory-acquired infections. Hands should be washed with an appropriate germicidal soap before exiting the laboratory and after infectious materials are handled. (Laboratorians working with gram-positive organisms should use alcohol (70%) to cleanse their hands if germicidal soap is unavailable.)

## Eating

Eating, drinking, and smoking are not permitted in laboratory work areas. Food must be stored and eaten outside of the work area in designated areas used for that purpose only. Personal articles (*e.g.*, handbags, eyeglasses, or wallets) should not be placed on the workstations.

## Mouth pipetting

Mouth pipetting is **strictly prohibited** in the laboratory. Rubber bulbs or mechanical devices should be used.

## Sharps

A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, pipettes, capillary tubes, and scalpels. Dispose of sharps in designated containers. To minimize finger sticks, used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal. Non-disposable sharps, including syringes, should be placed in a labeled discard pan for decontamination before cleaning. Broken glassware should not be handled directly by hand but should be removed by mechanical means (*e.g.*, a brush and dustpan, tongs, or forceps).

## Aerosols

All procedures must be carefully performed to minimize splashes or aerosolization. Techniques that tend to produce aerosols should be avoided. Inoculating wires and

loops should be cooled by holding them still in the air for 5 – 10 seconds before they touch colonies or clinical material. Loops containing infectious material should be dried in the hot air above a burner before flaming. Vortexing and centrifugation should be done in closed containers. (If safety capped tubes are not available, sealed tubes should be used.) Gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Needles should never be cut or removed from the syringe before autoclaving. All body fluids should be centrifuged in carriers with safety caps only.

When procedures with a high potential for creating infectious aerosols are conducted or when a procedure is used that can result in splashing or spraying of the face with infectious or other hazardous materials, laboratory work should be conducted in a safety cabinet or by laboratorians wearing the appropriate face-protection equipment (e.g., goggles, mask, face shield, or other splatter guards). Procedures that pose a risk may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient pressures, inoculating animals intranasally, and harvesting infected tissues from animals or eggs. Face protection should also be used when working with high concentrations or large volumes of infectious agents.

### Decontaminating bench tops and other surfaces

Bench tops should be wiped with a disinfectant (a phenolic disinfectant, 1% sodium hypochlorite [bleach], or 70% isopropyl alcohol) routinely after working with infectious agents or clinical specimens or after spills, splashes, or other contamination by infectious materials. Solutions of disinfectants should be maintained at the work station (see *Disinfectants*).

### Disposal of contaminated materials

All discarded plates, tubes, clinical samples, and other contaminated materials should be placed in disposal containers at each bench. Special disposal boxes must be used for sharps (e.g., syringes or broken glass) to minimize the risk of injury. Avoid overfilling such containers. Containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal.

### Autoclaving

An autoclave must be available for the BSL-2/3 laboratory and must be operated only by personnel who have been properly trained in its use. To verify that each autoclave is working properly, spore strips or other biological indicators designed to test for efficiency of sterilization should be included in autoclave loads on a

regular basis. Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or by other means (e.g., biological indicators).

### General laboratory policies

All areas of the laboratory must be kept clean and orderly. Dirt, dust, crowding, or clutter is a safety hazard and is not consistent with acceptable biological research. Floors should be kept clean and free of unnecessary clutter. They should be washed with a germicidal solution on a regular basis and after any spill of infectious material has occurred.

### Refrigerators and freezers

Refrigerators and freezers should be regularly inspected for the presence of broken vials or tubes containing infectious agents. When removing and discarding broken material, laboratorians should wear gloves and proper protective attire (e.g., laboratory coat, goggles, or face-shield). Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination and temperature failure.

### Fire prevention

Burners should be used away from lamps and flammable materials. Bulk flammable material must be stored in the safety cabinet. Small amounts of these flammable materials (e.g., ethyl acetate, ethyl alcohol, and methanol) can be stored in safety containers. Burners must be turned off when not in use. All laboratorians must know the location of fire extinguishers, fire blankets, and showers, and fire safety instructions and evacuation routes should be posted.

## Special practices

### Transport of biohazardous materials

Transport of biohazardous materials from one building to another increases the risk of breakage and spills. If transport is necessary, the primary infectious agent container (regardless of size) must be placed in an unbreakable second container that can be sealed (e.g., using a screw-top tube or a plastic bag).

### Disinfectants

Organisms may have different susceptibilities to various disinfectants. As a surface disinfectant, 70% alcohol is generally effective for the *Enterobacteriaceae*, but other organisms are more resistant. However, 70% isopropyl alcohol is not the

disinfectant of choice for decontaminating spills. Phenolic disinfectants, although expensive, are usually effective against many organisms. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy, especially before use on BSL-3 organisms (e.g., *Mycobacterium tuberculosis*). **A effective general disinfectant is a 1:100 (1%) dilution of household bleach (sodium hypochlorite) in water; at this dilution, bleach can be used for wiping surfaces of benches, hoods and other equipment.** A 1:10 (10%) dilution of bleach is corrosive and will pit stainless steel and should not be used routinely; however, the 10% bleach solution may be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred. If sodium hypochlorite is used as a disinfectant, the standard 1% dilutions should be made **daily** from a stock solution.

### Decontamination of spills

The following procedure is recommended for decontaminating spills.

- Isolate the area to prevent anyone from entering.
- Wear gloves and protective clothing (e.g., a gown or lab coat, shoes, and a mask [if the spill may contain a respiratory agent or if the agent is unknown]).
- Absorb or cover the spill with disposable towels.
- Saturate the towels with an appropriately diluted intermediate or high-level disinfectant (e.g., a phenolic formulation or household bleach).
- Place disinfectant-soaked towels over the area and leave them in place for at least 15 minutes before removing and discarding them.
- Wipe area using clean disinfectant-soaked towels and allow area to air dry.
- Place all disposable materials used to decontaminate the spill into a biohazard container.
- Handle the material in the same manner as other infectious waste.

### Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water for 15 minutes. In the event of a centrifuge accident in which safety carriers have not been used, other personnel in the area should be warned immediately and the area isolated to prevent anyone from entering.

## Protective clothing and equipment

### Laboratory coats

Protective coats, gowns, smocks, or uniforms designated for laboratory use must be worn while working in the laboratory. Laboratory coats should fit properly and should cover arms to the wrist. This protective clothing should be removed and left in the laboratory before leaving for non-laboratory areas. All protective clothing is either disposed of in the laboratory or laundered by the institution; it should never be taken home by personnel.

### Gloves

Regardless of the type of infectious material, gloves should be worn when performing potentially hazardous procedures (e.g., slide agglutination) in which there is a risk of splashing or skin contamination or when the laboratory worker has cuts or broken skin on his or her hands. Gloves should always be worn when handling clinical specimens, body fluids, and tissues from humans and animals. These tissues should be assumed to be positive for hepatitis B virus, human immunodeficiency virus (HIV), other bloodborne pathogens, and *M. tuberculosis*. Gloves must be removed when contaminated by splashing or spills or when work with infectious materials is completed. Gloves should not be worn outside the laboratory. Personnel should not use the telephone or open doors with gloves that have been used in laboratory procedures. All used gloves should be disposed of by discarding them with other disposable materials and autoclaving. **Hands should be washed immediately after removing gloves.**

### Barrier precautions

Clinical specimens, body fluids, and tissues from humans and animals should be assumed to be positive for hepatitis B virus, HIV, other bloodborne pathogens, and *M. tuberculosis*. These materials should be handled in a safety cabinet or using other barrier precautions (e.g., goggles, mask, face shield, or other splatter guards) whenever a procedure is performed that can potentially create an aerosol.

## Useful biosafety references for the laboratory

- Centers for Disease Control and Prevention, National Institutes of Health. *Biosafety in microbiological and biomedical laboratories*. Washington, DC: U.S. Government Printing Office; 1999: stock no. 017-040-00547-4.
- World Health Organization. *Laboratory Biosafety Manual*, 2nd edition. Geneva: WHO; 1993: ISBN 92 4 154450 3.

## Media, Reagents, and Quality Control

Each laboratory must ensure adequate control of the media and reagents it uses. Quality control (QC) includes the selection of satisfactory reagents, the preparation of media according to approved formulations or specific manufacturer's instructions, and the use of well-characterized reference strains to check prepared media. The World Health Organization (WHO) encourages central public health laboratories to participate in at least three external quality assessment surveys per year; for reference laboratories, this may involve an international testing scheme. National central laboratories (reference laboratories) should work to standardize procedures of regional and local laboratories to their own, so that observations can be interpreted in the same manner across sites.

### Quality control of media

A summary of considerations for quality control of media, methods, and sources of quality control strains follows:

#### 1) Considerations for quality control of media

Each batch of medium prepared from individual ingredients or each different manufacturer's lot number of dehydrated medium should be tested for sterility, the ability to support growth of the target organism(s), and/or the ability to produce appropriate biochemical reactions, as appropriate.

##### ***Sterility***

- Incubate one tube or plate from each autoclaved or filter-sterilized batch of medium overnight at 35°–37°C and examine it for contaminants.

##### ***Ability to support growth of the target organism(s)***

- Use at least one strain to test for ability of selective media to support growth of the target pathogen (e.g., for MacConkey agar, a *Shigella* strain such as *S. flexneri*). Documentation should be made regarding whether this strain produces the appropriate biochemical reactions / color on the test medium.

(Discussions of specific biochemical reactions are included in the media section of this appendix.)

### ***Ability to produce appropriate biochemical reactions***

- **For selective media:** Use at least one organism that will grow on the medium and at least one organism that will not grow on the selective medium to test for the medium's ability to differentiate target organisms from competitors. If the medium is both selective and differential, it may be useful to include two organisms that will grow on the medium and produce different reactions (e.g., for MacConkey agar: a lactose-nonfermenting organism such as *S. flexneri*; a lactose-fermenting organism such as *E. coli*; and, *S. aureus*, which should not grow).
- **For biochemical media:** Use at least one organism that will produce a positive reaction and at least one organism that will produce a negative reaction (e.g., for urea medium, a urease-positive organism such as *Proteus* and a urease-negative organism such as *E. coli*).

## **2) Methods for quality control of media**

When testing for ability of a medium to support growth, a small inoculum will give greater assurance that the medium is adequate for recovery of a small number of organisms from a clinical specimen; therefore, use a dilute suspension of control organisms to inoculate the medium for QC. An example of a protocol for quality control of media follows here:

- a) Inoculate the control strain to nonselective broth (e.g., a tryptone-based soy broth [TSB]) and incubate / grow overnight.
- b) Prepare a standardized inoculum for testing the medium. The appropriate standard dilution differs for selective and nonselective media.
  - **If testing selective or inhibitory media:** To prepare a standardized inoculum for testing selective and inhibitory media, make a 1:10 dilution of the overnight nonselective broth culture.
  - **If testing nonselective media:** To prepare a standardized inoculum for testing nonselective media, make a 1:100 dilution of the nonselective broth culture.
- c) Using a calibrated loop, if available, inoculate one tube or plate of each medium with a loopful of the standardized inoculum of the control strain(s). (If performing QC of plating medium, streak for isolation.) The same loop should be used for all QC of all media; it is more important to have the consistency of the same inoculating loop every time than it is to use a calibrated loop.

- **If testing selective or inhibitory media:** A nonselective plating medium (e.g., heart infusion agar [HIA]) should be inoculated at the same time as the selective medium for comparison purposes.

### 3) Sources of quality control strains

Suitable QC strains may be obtained in the following ways.

- A laboratory may use strains isolated from clinical specimens or quality assurance specimens, provided the strains have been well characterized by all available methods (e.g., biochemical, morphologic, serologic, and molecular).
- Many laboratories purchase QC strains from official culture collections (e.g., the American Type Culture Collection [ATCC] and the National Collection of Type Cultures [NCTC]).<sup>34</sup> (Addresses for ATCC and NCTC are included in Appendix 13.)

***Quality control strains appropriate for antimicrobial susceptibility testing as included in this manual***

The following ATCC numbers can be used to identify the appropriate antimicrobial susceptibility testing QC organisms included in this laboratory manual.

<i>Haemophilus influenzae</i>	<i>H. influenzae</i> ATCC 49247 * (* for testing of the antimicrobial agents included in this laboratory manual)
<i>Neisseria meningitidis</i>	<i>S. pneumoniae</i> ATCC 49619
<i>Streptococcus pneumoniae</i>	<i>S. pneumoniae</i> ATCC 49619
<i>Neisseria gonorrhoeae</i>	<i>N. gonorrhoeae</i> ATCC 49226 (other reference strains, available from CDC, may be used when testing antimicrobial agents not included in NCCLS criteria)
<i>Salmonella</i> serotype Typhi	<i>E. coli</i> ATCC 25922
<i>Shigella</i>	<i>E. coli</i> ATCC 25922
<i>Vibrio cholerae</i>	<i>E. coli</i> ATCC 25922

<sup>34</sup> This manual presents the ATCC numbers for quality control organisms, but these ATCC strains may also be obtained from the NCTC.

# Quality control of reagents

As with all other products used in testing, reagents (whether purchased or prepared in the laboratory) should be clearly marked to indicate the date on which they were first opened and the expiration date, if appropriate. Each reagent should be tested to make sure the expected reactions are obtained.

If the reagent is a rare, expensive, or difficult-to-obtain product (e.g., diagnostic antiserum) it does not necessarily have to be discarded on the expiration date. If satisfactory sensitivity and specificity can still be verified by normal QC procedures, the laboratory may indicate on the vial label the date of verification of quality of the reagent. All reagents should be tested for quality at intervals established by each laboratory to ensure that no deterioration has occurred; if the quality of the reagent is being verified after the expiration date, testing should be performed more frequently.

## Slide agglutination method for quality control of antisera

For QC of antiserum, two or more control strains (one positive and one negative) should be used to test the agglutination characteristics of the antiserum. The results of all reactions should be recorded. Following is an example of a typical QC procedure.

- Place a drop (about 0.05 ml, though as little as 10 µl can be used) of each antiserum on a slide or plate. Also, place a drop of 0.85% saline on each slide or plate to test each antigen for roughness or autoagglutination.
- Prepare a densely turbid suspension (2 or 3 McFarland turbidity standard, see Table 21) of each control isolate in 0.85% saline with growth aseptically harvested from an 18- to 24-hour culture from nonselective agar (e.g., HIA or tryptone soy agar [TSA]).
- Add one drop of the antigen suspension to the antiserum and the saline. Mix thoroughly with an applicator stick, glass rod, or inoculating loop. Rock the slide back and forth for 1 minute.
- Read the agglutination reaction over a light box or an indirect light source with a dark background. The saline control must be negative for agglutination for the test to be valid.

The degree of agglutination should be read and recorded as follows:

Percentage of agglutination:	Record reaction as:
100%	4+
75%	3+
50%	2+
25%	1+
0%	negative

## Advantages of centralized acquisition of media and reagents

Centralizing acquisition of media and reagents in the national reference laboratory or Ministry of Health can provide several benefits:

- Large amounts of a single lot of medium or reagent can be purchased and subsequently divided into smaller aliquots for distribution to provincial/district laboratories. This may be more cost effective because of, *e.g.*, discounts for larger orders, lower shipping costs, less waste because of product going past expiration date, etc.
- Quality control can be performed in the central laboratory, avoiding duplication of effort among provincial and district laboratories. An unsatisfactory medium or reagent can then be returned to the manufacturer before the lot is distributed to other laboratories.
- The standardization of methods among laboratories at all levels is facilitated by use of single lots of media.

## Preparation of media and reagents

Each manufacturer's lot number of commercial dehydrated medium and each batch of medium prepared from individual ingredients should be quality controlled before use. Immediately after preparation, each medium should be tested, as appropriate, with a reference strain of *H. influenzae*, *N. meningitidis*, *S. pneumoniae*, *N. gonorrhoeae*, *S. Typhi*, *Shigella*, and/or *V. cholerae* O1/O139 for proper growth characteristics as described for each medium.

A record of all media preparation or purchase dates and quality control test results should be kept, and any unusual characteristic (*e.g.*, the color of the medium or slow growth of test bacteria) should be noted.

Many media call for the use of defibrinated blood. Defibrinated blood can be prepared mechanically by swirling 30 ml blood and (*e.g.*) sterile glass beads or a wooden-stick device in a 125–250 ml Erlenmeyer flask at approximately 90 rpm for 7–9 minutes. (Sterile paper clips in a flask can also serve to assist in defibrination.) Blood is defibrinated when clotting factors have been removed; they will be visible in the flask as a translucent, fibrous “web.” (A useful reference for “low-technology” methods to defibrinate blood is in a publication by Kay *et al.* [1986], and is included in Appendix 15.)

Agar media should be dispensed into 15 x 100-mm or 15 x 150-mm Petri dishes to a uniform depth of 3–4 mm; approximately 20-ml of liquid agar medium will achieve this depth in a 15 x 100-mm plate. If agar is cooled to 50°C prior to pouring, condensation is minimized. After pouring, the plates should be kept at

room temperature for several hours to prevent excess condensation from forming on the covers of the dishes. Another means by which condensation will be reduced is if plates are stacked so that they cool more slowly. Alternatively, if when preparing **selective** media (e.g., MacConkey [MAC], xylose lysine desoxycholate [XLD], thiosulfate citrate bile salts [TCBS] agar, etc.), conditions are such that there is little chance that the cooling media will be contaminated, after the agar is poured into the plates, the lids can be placed on the dish so that a small opening is left to let the heat out, resulting in the formation of less condensation on the upper lid; the lid should remain slightly open like this for approximately 30 minutes, while the agar solidifies. If, however, it is likely that the agar will be contaminated if the lid is left partly open, the agar should be allowed to solidify with the lid closed.

**Note:** Covering the agar while it is still hot will allow for the formation of a substantial amount of condensation on the upper lid. If the plates contain condensation, the plates should be covered at room temperature for 24 hours to allow the condensation to evaporate. After condensation has evaporated, the plates should be placed in an inverted position and stored in a plastic bag in an inverted position at 4°C.

**Media for enrichment, identification, and antimicrobial susceptibility testing**

Some of the culture media included in this manual are commonly referred to by their abbreviations rather than their full names. Therefore, when these media are mentioned in the methods for the preparation or quality control of other media in this section, they are referred to by their abbreviations.

Name of commonly abbreviated culture medium	Abbreviation
Alkaline peptone water	APW
Bismuth sulfite agar	BS
Cystine trypticase agar	CTA
Desoxycholate citrate agar	DCA
Gram-negative broth	GN
Hektoen enteric agar	HE
Heart infusion agar	HIA
<i>Haemophilus</i> test medium	HTM
Kligler iron agar	KIA
Lysine iron agar	LIA
MacConkey agar	MAC
Martin-Lewis agar	ML
Modified Thayer-Martin agar	MTM

## Name of commonly abbreviated culture medium, *continued*      Abbreviation

Phosphate buffered saline	PBS
Selenite broth	SEL
Sulfide-indole-motility medium	SIM
<i>Salmonella-Shigella</i> agar	SS
Thiosulfate citrate bile salts sucrose agar	TCBS
Tryptone (Trypticase) soy agar	TSA
Tryptone (Trypticase) soy broth	TSB
Triple sugar iron agar	TSI
Xylose lysine desoxycholate agar	XLD

Methods for the preparation of media (from individual ingredients or commercially available preparations) follow, in alphabetical order.

### Acidometric agar

This agar may be used to test *H. influenzae* for  $\beta$ -lactamase if nitrocefin is not available.

Distilled water	100 ml
Agar	1.5 g
0.5% phenol red solution	0.2 ml
NaOH (1 Normal)	
Penicillin G powder (to prepare a 5000 unit/ml concentration)	

Combine distilled water, agar, and phenol red solution and boil until the agar dissolves. Adjust the pH of the solution with NaOH until the pH is in the range of 8.5 – 9.0. Dispense into 20-ml tubes and cool in a water bath to 50°–55°C. Add penicillin G powder (to yield a concentration of 5000 unit/ml) to the tube and vortex (or mix well). Re-check the pH for the proper range, pour the contents of the tube into a Petri plate and allow it to solidify.

### Quality control:

- The acidometric agar surrounding  $\beta$ -lactamase-positive colonies will be yellow in color after incubation for one hour at 35°C.
- The acidometric agar surrounding  $\beta$ -lactamase-negative colonies will exhibit no color change after incubation for one hour at 35°C.

### Alkaline peptone water (APW)

Alkaline peptone water (APW) can be used to enhance the recovery of *V. cholerae* when there are few organisms present. [**Note:** There are several different published formulations for this medium.]

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Add ingredients to the water and adjust to pH 8.5 with 3 M NaOH solution. Distribute into jars, bottles, or tubes, and autoclave at 121°C for 15 minutes. Store at 4°C for up to 6 months, making sure containers' caps are tightly closed to prevent a drop in pH or evaporation.

- **Peptone water** differs from APW in that it does not have added salt, nor is the pH adjusted to 8.5. Add 10 g of peptone to 1000 ml of distilled water; distribute, autoclave and store as previously described for APW.

**Quality control:** When inoculated into alkaline peptone water for quality control, *V. cholerae* O1 should show good growth at 6–8 hours.

### Bismuth sulfite agar (BS)

Bismuth sulfite agar (BS), which is highly selective, is the preferred medium for isolation of *S. Typhi*. However, **bismuth sulfite agar should not be used for isolation of *S. Typhi* if it has been stored for more than 24–36 hours**. BS has been reported to inhibit *Salmonella* serotypes other than *S. Typhi* unless it is refrigerated at 4°C for at least 24 hours before use. When culturing fecal specimens from suspected typhoid carriers, the use of a BS pour plate may enhance isolation.

Prepare according to manufacturer's instructions. [**Note:** Several commercial brands of bismuth sulfite agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.] Heat to boiling to dissolve, but avoid overheating (*i.e.*, once dissolved, remove from heat). Do not autoclave.

*For preparation of BS streak plates:*

When cool enough to pour, dispense the BS medium into plates. Plates can be stored at 4°C for up to 1 week if BS will not be used to isolate *S. Typhi*.

*For preparation of BS pour plates:*

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.

**Quality control:** The following organisms should be adequate for quality control of BS agar:

- *S. Typhi* should produce excellent growth of black colonies with a metallic sheen;

- *E. coli* should grow poorly, if at all, and will appear as brown to green colonies.

### Blood agar: TSA with 5% sheep blood

Sheep blood agar is used as a general blood agar medium, and consists of TSA plus 5% sheep's blood. The sheep blood agar plate should appear a bright red color. If the plates appear dark red, the blood has been added when the agar was too hot; if this happens, the medium should be discarded and a new batch prepared.

- Prepare TSA according to the instructions given on the label of the dehydrated powder. For convenience, 500 ml of molten agar can be prepared in a 1-liter flask. Add 20 g of agar into 500 ml of water. Heat to dissolve.
- Autoclave at 121°C for 20 minutes. Cool to 60°C.
- Add 5% sterile, defibrinated sheep blood (*i.e.*, add 25 ml sheep blood to 500 ml of agar). If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (*e.g.*, 50 ml of blood per liter of medium).
- Dispense 20 ml into 15 x 100-mm Petri dishes. Allow the medium to solidify and dry out, place in a plastic bag, and store at 4°C.

**Quality control:** Test each new, freshly prepared or purchased batch of blood agar plates for growth and hemolytic reaction with a strain of *S. pneumoniae*. The colonies are small and should appear grey to grey-green surrounded by a distinct greenish halo in the agar (Figure 56).

### Blood culture broth

Blood culture broth contains TSB and sodium polyanetholesulfonate (SPS).

- Follow the instructions of the manufacturer on the label of each bottle of dehydrated trypticase soy broth.
- Add 0.25 g SPS per liter of medium. SPS is especially important for recovery of *H. influenzae* since it prevents the inoculated blood from clotting.
- Dispense in 20-ml (pediatric blood culture bottle) and 50-ml (adult blood culture bottle) amounts into suitable containers (*e.g.*, tubes or bottles) with screw-caps with rubber diaphragms. The amount of liquid in the containers should comprise at least two-thirds of the total volume of the container.
- Sterilize by autoclaving at 121°C for 15 minutes. Cool and store medium at room temperature.

**Quality control:** Each new batch of freshly prepared or purchased blood culture medium should be tested for supporting the growth of a variety of pathogens

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

### Chocolate agar with TSA base and growth supplement

Chocolate agar with growth supplements is a medium that supports the special growth requirements needed for the isolation of fastidious organisms (when incubated in a 5% CO<sub>2</sub> atmosphere). Chocolate agar contains a reduced concentration of agar, which increases the moisture content of the medium.

**Supplemented chocolate agar should support the growth of *H. influenzae*.**

Chocolate agar slants for transport and short-term storage can be prepared in the same manner as that described for agar plates, except that the medium is dispensed in 16 x 125-mm screw-cap tubes and slanted before solidifying.

- a) Use TSA as the basal medium. Prepare double strength (i.e., 20 g in 250 ml distilled water). Autoclave, and then cool to 50°C. Use the thermometer to verify the cooling temperature.
- b) Prepare a solution of 2% hemoglobin (i.e., 5 g in 250 ml distilled water). Mix the hemoglobin in 5–6 ml of the distilled water to form a smooth paste. Continue mixing as the rest of the water is added. Autoclave, and cool to 50°C.
- c) Add the hemoglobin solution to the double-strength TSA and continue to hold at 50°C.
  - **Alternative to steps a-c:** If a hemoglobin solution is unavailable, an alternative is to add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (i.e., 5 ml blood per 100-ml agar) to full-strength TSA (i.e., 20 g in 500 ml distilled water). **DO NOT use human blood.** After the base medium has been autoclaved and cooled to 50°C, add the blood and place in a hot water bath at no more than 80°C for 15 minutes or until a chocolate color is achieved. Then cool to 50°C.
- d) After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add growth supplement (e.g., IsoVitaleX or Vitox) to a final concentration of 1%. Mix the ingredients by gently swirling the flask; avoid forming bubbles.
- e) Dispense 15–20 ml in each 15 x 100-mm Petri dish.

**Quality control:** All freshly prepared or purchased chocolate agar media should be tested to determine the medium's capacity to support growth of the bacteria to be isolated, particularly *H. influenzae*. If the medium does not support the growth of one or all of the bacteria, the medium should be discarded, and a new batch of medium should be prepared or purchased.

- Chocolate agar should look brown to brownish-red in color. *N. meningitidis* and *H. influenzae* should appear as a greyish, almost translucent film on the slant's surface with no discoloring of the medium after 24 hours of incubation; *S. pneumoniae* should appear as small grey to grey-green colonies with a very distinct greenish discoloring of the medium (see Figures 61 and 62).

**If *H. influenzae* does not grow, the growth supplement (IsoVitaleX or its equivalent) may have been inadvertently omitted.**

### Chocolate agar with bacitracin

This medium is used for primary isolation of *H. influenzae* from respiratory sources. **Bacitracin-chocolate agar should not be used for subcultures.**

A stock solution of bacitracin can be made by suspending 3 g of bacitracin in 20 ml of distilled water. The solution should be sterilized by filtration. Dispense in 1-ml amounts and store in a -20°C to -70°C freezer.

- a) Prepare chocolate agar suspension according to the instructions for the preparation of chocolate agar outlined above (in steps a–d).
- b) After adding the hemoglobin and growth supplement to the TSA base medium, cool the medium to 50°C, and add 1.0 ml of stock solution of bacitracin per 500 ml of chocolate agar.
- c) Dispense 15–20 ml in each 15 x 100-mm Petri dish (so that agar is a uniform 3–4 mm); this volume makes 25 to 30 bacitracin-chocolate agar plates.
- d) After quality control testing, the plates should be placed in plastic bags and stored at 4°C.

The final concentration of bacitracin in the chocolate agar is 300 µg/ml. The bacitracin does not change the color of the medium, and it should appear brown like standard supplemented chocolate agar (similar to the medium shown in Figure 58).

**Quality control:** For quality control of bacitracin-chocolate agar, a strain of *H. influenzae* (e.g., ATCC 49247) should be tested for proper growth characteristics.

### Cystine trypticase agar (CTA) with 1% carbohydrate

CTA medium is a semi-solid medium presented in this manual for the biochemical testing of *N. meningitidis* with glucose, maltose, lactose, and sucrose. It can be purchased as a ready-made medium or prepared from dehydrated media with the

addition of sugars. (Follow the manufacturer's instructions when using dehydrated medium.)

- a) Suspend 28.0 g of cystine trypticase agar medium in 900 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
- b) Autoclave the flask at 121°C for 15 minutes. Cool to 50°C.
- c) Prepare a 10% glucose solution using 10 g of glucose in 100 ml of distilled water. Filter-sterilize the solution using a 0.22-micron filter.
- d) Aseptically add this entire solution (100 ml of 10% glucose solution) to the 900 ml of the CTA medium to obtain a final 1% concentration of the glucose.
- e) Dispense 7 ml into each 16 x 125-mm glass screw-capped tube.
- f) Store at 4°C.
- g) Repeat this procedure for the remaining three carbohydrates (in place of glucose in step c): maltose, lactose, and sucrose.

**Quality control:** Testing of CTA agar with a reference strain of *N. meningitidis* will yield an acid reaction (indicated by a color change from red to yellow) in glucose and maltose (but not in lactose or sucrose). Reactions typical of other organisms are included in Table 3. It should be noted that because *N. gonorrhoeae* typically has a weak glucose reaction difficult to detect in CTA media, this laboratory manual suggests using a rapid acid-detection test (in place of CTA) for presumptive *N. gonorrhoeae*.

### Desoxycholate citrate agar (DCA)

Desoxycholate citrate agar (DCA) is a differential selective plating medium for the isolation of enteric pathogens, particularly *Shigella* and *Salmonella*. Lactose-fermenting organisms produce pink colonies surrounded by a zone of bile precipitation. Colonies of lactose-nonfermenting strains are colorless. Several formulations of DCA, which may vary in selectivity, are available from different manufacturers.

Prepare according to manufacturer's instructions. [**Note:** DCA may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when prepared from commercial dehydrated preparations.] DCA medium is very heat-sensitive, and overheating during boiling should be avoided; **do not autoclave DCA medium**. Plates can be stored at 4°C for up to a week.

**Quality control:** For quality control of DCA, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* may be somewhat inhibited, depending on the particular formulation used, but will produce pink colonies surrounded by a zone of precipitated bile; and,
- *S. flexneri* and *S. dysenteriae* 1 will produce fair-to-good growth of colorless colonies.

### Formalinized physiological saline

(Refer to “Physiological saline,” listed later in this Appendix.)

### GC-Chocolate agar (gonococcus agar medium [GC] base plus hemoglobin and 1% defined growth supplement)

GC-chocolate agar is a nonselective medium used to grow pure cultures of *Neisseria gonorrhoeae*. Although chocolate agar is often made with fresh sheep blood, it is **not** recommended that fresh blood products be used in gonococcal medium because they vary in their ability to support the growth of *N. gonorrhoeae*. Hemoglobin powder must therefore be used to prepare a standardized GC-chocolate agar medium.

The following methods allow for the production of 100 ml of medium (five 15 x 100-mm diameter plates); adjust quantities proportionately for the production of larger volumes of media. It is suggested that laboratorians should prepare no more than 500 ml of media per individual container, because it is difficult to properly mix larger amounts of media.

- Suspend 7.2 g of GC agar base in 100 ml of distilled water. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder (approximately 1 minute). Autoclave the medium at 121° C for 15 minutes. Cool in a water bath set to 50° C.
- Add 2 g of soluble hemoglobin powder to 5–10 ml of warm distilled water in a screw-cap bottle. Gradually add warm distilled water (to a total volume of 100 ml) and gently agitate the bottle until a smooth suspension is achieved. (This process is made easier if the solution can be stirred using a magnetic stirrer.) Autoclave the hemoglobin solution at 121°C for 15 minutes. Cool in a water bath set to 50°C.
  - If ready-made sterile hemoglobin solution is available, instead of following the methods described here in step b, use 100 ml of ready-made 2% sterile hemoglobin solution warmed in a water bath set at 50°C.
- Reconstitute the growth supplement (e.g., IsoVitaleX).
- Aseptically open the vial containing the lyophilized growth supplement.
- Use a sterile needle and syringe to aseptically transfer 10 ml of the accompanying diluent to the vial.

- f) Shake to ensure complete solution. After reconstitution, use growth supplement immediately, or store at 4°C and use within 2 weeks.
- g) Aseptically add 100 ml sterile hemoglobin solution and 2 ml of reconstituted growth supplement to 100 ml of GC agar base medium. Mix gently, but thoroughly, to avoid the formation of air bubbles in the agar (*i.e.*, foam). Holding the bottle in an upright position, bottle neck in the hand, and gently swirling it three times in one direction (*e.g.*, clockwise) and then three times in the other direction (*e.g.*, counter-clockwise) is a good, gentle mixing technique.
- h) Dispense 15–20 ml volumes of medium into each sterile 15 x 100-mm Petri dish to achieve a uniform depth of 3–4 mm in the plate.
- i) Replace the lid on the plate, and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4°C.

**Quality control:** Inoculated GC-chocolate agar should support the growth of *N. gonorrhoeae* (*e.g.*, the fastidious strain 14AHU [available from CDC, see Appendix 14] as well as ATCC 49226) after incubation at 35°–36.5°C in a 5% CO<sub>2</sub>, humid atmosphere for 18–24 hours. QC strains may be inoculated onto either whole- or half-plates.

#### GC- susceptibility test medium (GC-agar base medium plus 1% defined growth supplement)

**Mueller-Hinton agar does not support growth of *N. gonorrhoeae***, and therefore must not be used for antimicrobial susceptibility testing of this organism.

**Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on a GC-susceptibility test medium**, a simple nonselective medium of GC agar base plus 1% defined supplement (*e.g.*, IsoVitaleX). GC-susceptibility test medium does not contain hemoglobin (*i.e.*, it is not a chocolate medium), unlike other GC test media.

GC-susceptibility test medium is similar to but simpler than the standard GC-chocolate medium; it is prepared in the same manner with the exception of the exclusion of hemoglobin from the GC-susceptibility testing medium. The methods described here allow for the production of 100 ml of medium (five 15 x 100-mm diameter plates); adjust quantities proportionately for the production of larger volumes of media. If a large number of plates is required, prepare no more than 500 ml of media per individual container (because it is difficult to mix larger quantities of the ingredients properly).

- a) Suspend 7.2 g of GC agar base in 100 ml of distilled water. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder (approximately 1 minute). Autoclave the medium at 121° C for 15 minutes. Cool in a water bath set to 50° C.

b) Reconstitute the growth supplement (e.g., IsoVitaleX).

- 1) Aseptically open the vial containing the lyophilized growth supplement.
- 2) Use a sterile needle and syringe to aseptically transfer 10 ml of the accompanying diluent to the vial.
- 3) Shake to assure complete solution. After reconstitution, use growth supplement immediately, or store at 4°C and use within 2 weeks.

c) Aseptically add 2 ml of reconstituted growth supplement to 100 ml of GC agar base medium. Mix gently, but thoroughly, to avoid the formation of air bubbles in the agar (i.e., foam). Holding the bottle in an upright position, bottle neck in the hand, and gently swirling it three times in one direction (e.g., clockwise) and then three times in the other direction (e.g., counter-clockwise) is a good, gentle mixing technique.

d) Dispense 15–20 ml volumes of medium into each sterile 15x100-mm Petri dish to achieve a uniform depth of 3–4 mm in the plate.

e) Replace the lid on the plate, and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4°C.

**Quality control:** Each new lot of GC-susceptibility test medium should be quality controlled by testing both the NCCLS-recommended *N. gonorrhoeae* strain (ATCC 49226) and the other reference strains to be used when performing antimicrobial susceptibility testing of *N. gonorrhoeae*. Appropriate ranges of antimicrobial susceptibility test values for these QC strains are listed in Tables 9 and 10.

## Gonococcal selective media

Gonococcal selective media support adequate growth of *N. gonorrhoeae* from clinical specimens while inhibiting commensal species and fungi. A variety of media share the same supplemented GC-chocolate base and antibacterial agents (vancomycin and colistin), and vary by antifungal agents (e.g., anisomycin or nystatin) and additional antibacterial agents (e.g., amphotericin B or trimethoprim lactate). *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and *K. denitrificans* are routinely able to grow on gonococcal selective media whereas strains of most commensal species cannot. Two commonly used selective gonococcal media mentioned in this manual are Modified Thayer-Martin and Martin-Lewis.

**Modified Thayer-Martin (MTM)** is GC-chocolate agar containing 3 µg/ml vancomycin, 7.5 µg/ml colistin, 12.5 units/ml nystatin and sometimes also 5 µg/ml trimethoprim lactate. Similarly, **Martin-Lewis (ML)** is GC-chocolate agar containing 4 µg/ml vancomycin, 7.5 µg/ml colistin, 20 µg/ml anisomycin and sometimes 5 µg/ml trimethoprim lactate. The antimicrobial/antifungal agents are commercially available as combined inhibitory supplements (e.g., “VCA”, “VCN”, “VCNT”, and GC-supplement).

Laboratories able to access inhibitory supplements can prepare their own gonococcal selective media by following the instructions included in this manual for the supplemented GC-chocolate base and following the manufacturer's instructions for the addition of the inhibitory supplement to the base medium. Alternatively, laboratories can purchase prepared gonococcal selective media from commercial suppliers.

**Quality control:** Gonococcal selective media should support the growth of *N. gonorrhoeae* (e.g., ATCC 49226) after incubation at 35°–36.5°C in a 5% CO<sub>2</sub>, humid atmosphere for 18–24 hours. *N. cinerea* is colistin-susceptible and should **not** grow on gonococcal selective media. Additional QC strains may be suggested on the manufacturer's package insert.

- **Note:** Because some gonococcal strains are vancomycin-sensitive, part of the quality assurance program should include comparing the rate of positive culture results with the rate of observation of gram-negative diplococci in urethral smears from the corresponding men with uncomplicated symptomatic gonorrhea. If a discrepancy is observed between these rates, laboratorians should culture the next (approximately) 50 specimens first on nonselective GC-chocolate agar and then on the selective medium. If cultures grow on the nonselective medium and exhibit poor growth or no growth on the selective medium, laboratorians should suspect that isolates are susceptible to vancomycin and consider sending strains to a reference lab for confirmation.

### Gram-negative (GN) broth

Gram-negative (GN) broth is a selective / inhibitory medium for isolation of gram-negative organisms. It may be used for the enrichment of fecal specimens suspected to contain *Salmonella* serotype. Prepare GN broth according to manufacturer's instructions. [**Note:** GN broth may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when prepared from commercial dehydrated preparations.]

**Quality control:** After overnight enrichment in GN broth, *S. Typhi* should produce good growth of colorless colonies on MacConkey agar.

### *Haemophilus* test medium (HTM)

The unsupplemented Mueller-Hinton agar used for antimicrobial susceptibility testing of many bacteria included in this manual does not support growth of *H. influenzae* and therefore must not be used for the antimicrobial susceptibility testing of this organism. **Antimicrobial susceptibility testing of *H. influenzae* is performed on *Haemophilus* test medium (HTM).**

HTM can be prepared by supplementing thymidine-free Mueller-Hinton agar with 15 µg/ml β-NAD (β- nicotinamide adenine dinucleotide), 15 µg/ml bovine haematin and 5 mg/ml yeast extract. However, to decrease lot-to-lot variation, **it is suggested that laboratories prepare HTM from commercially available dehydrated preparations when possible** (or purchase ready-made plated media).

Prepare HTM according to manufacturer's instructions.

**Quality control:** Incubate HTM for 48 hours at 35°C, and then for 120 hours at room temperature to ensure purity in the closed medium container. Each new lot of HTM should also be quality controlled by testing the NCCLS-recommended *H. influenzae* strain (ATCC 49247). Appropriate ranges of antimicrobial susceptibility test values for this QC strain are listed in Table 2.

### Heart infusion agar (HIA)

Heart infusion agar (HIA) is a general-purpose medium used with or without blood for isolating and cultivating a number of microorganisms. The medium should appear straw colored (a yellowish to gold coloring). HIA can also be used for determining the X- and V-factor requirements of *H. influenzae*.

- a) Prepare the HIA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks. These media should be fully dissolved with no powder on the walls of the vessel before autoclaving; stirring over heat may help the powder dissolve more rapidly.
- b) Autoclave at 121°C for 20 minutes.
- c) Cool to 50°C and pour into 15 x 100-mm Petri dishes.
- d) Allow medium to solidify and condensation to dry out before placing plates in plastic bags and storing at 4°C until used.

**Quality control:** Each freshly prepared or purchased batch of HIA should be quality control tested by determining the X and V requirements of *H. influenzae*. Inoculate a fresh plate of HIA with a control strain, such as *H. influenzae* ATCC 49247 (which should be readily available in laboratories performing antimicrobial susceptibility testing of *H. influenzae*); X, V, and XV disks should be placed on the inoculated plate identical to that shown in Figure 3. *H. influenzae* should grow only around the XV disk.

### Heart infusion rabbit blood agar (HIA-Rabbit blood)

HIA-rabbit blood is used for determining the hemolytic reaction of *Haemophilus* species. This medium should appear bright red and look very similar to blood agar plates. (Be sure to label the prepared medium carefully.) If the medium is dark red, discard and prepare a new batch. (Horse blood may be substituted for rabbit blood

in this medium; the preparation is exactly the same, with the exception of the blood source.)

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

**Quality control:** A strain of *H. haemolyticus* should be used to quality control the proper growth and hemolytic reactions of the HIA-rabbit blood medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete hemolysis that appears as a clear halo surrounding the colonies.

### Hektoen enteric agar (HE)

Hektoen enteric (HE) agar is a differential selective agar that is useful for isolation of *Salmonella* and *Shigella*. It has an H<sub>2</sub>S-indicator system for selecting H<sub>2</sub>S-producing *Salmonella*, which produce blue-green colonies with a black center. *Shigella* colonies are green whereas rapid lactose-fermenters (e.g., *E. coli*) are pink to orange with a zone of bile precipitation.

Prepare HE according to manufacturer's instructions. [**Note:** Several commercial brands of Hektoen enteric agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable when the medium is prepared in this manner than with a commercial dehydrated formulation.] Heat to boiling to dissolve, but avoid overheating (i.e., remove from heat after the powder has dissolved); do not autoclave. When cool enough to pour, dispense the HE into plates. Plates can be stored at 4°C for up to 1 week.

**Quality control:** For quality control of Hektoen enteric agar, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* should produce colonies that are pink to orange surrounded by a bile precipitate; and,
- *S. flexneri* should produce fair to good growth of green colonies, but *S. dysenteriae* 1 colonies should be smaller.

### Horse blood agar (blood agar base)

Horse blood agar is a highly nutritive medium that may be used for the primary isolation of *H. influenzae* and for the determination of the hemolysis with *H. haemolyticus* or other bacteria.

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

**Quality control:** The quality control testing of this medium is the same as that described for HIA-rabbit blood: a strain of *H. haemolyticus* should be used to quality control the proper growth and hemolytic reactions of horse blood agar medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete hemolysis that appears as a clear halo surrounding the colonies.

### Kligler iron agar and triple sugar iron agar

Kligler iron agar (KIA) and triple sugar iron (TSI) agar are carbohydrate-containing screening media widely used for identification of enteric pathogens. Both media differentiate lactose fermenters from nonfermenters and have a hydrogen sulfide indicator. H<sub>2</sub>S-producing organisms will cause blackening of the medium in both KIA and TSI.

KIA contains glucose and lactose. Organisms that ferment glucose cause the butt of the tube to become acid (yellow); some also produce gas. Lactose-fermenting organisms will produce an acid (yellow) slant; lactose-nonfermenting organisms will have an alkaline (red) slant.

TSI contains sucrose in addition to the ingredients in KIA. Organisms that ferment either lactose or sucrose will produce an acid (yellow) slant while organisms that ferment neither carbohydrate will have an alkaline (red) slant. As in KIA, in TSI glucose-fermenters produce an acid (yellow) reaction in the butt (sometimes with gas produced).

- a) Prepare according to manufacturer's instructions. [**Note:** There are several commercially available dehydrated formulations of KIA and TSI. These media can also be prepared from individual ingredients, but doing so may result in lot-to-lot variation.]
- b) Dispense a quantity of medium in appropriate tubes with a sufficient volume to give a deep butt and a long slant (e.g., dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes).
- c) Leave screw-caps loose, and autoclave the medium.
- d) After autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is about 3.5-cm deep and the slant is about 2.5-cm long.

- e) Tighten the screw-cap tops of the tubes and store at 4°C for up to 6 months.

**Quality control:** For quality control of KIA or TSI, the following organisms should be adequate for confirmation of biochemical response characteristics:

- *E. coli* should give an acid slant and butt, with the production of gas but no H<sub>2</sub>S;
- *S. flexneri* should give an alkaline slant, acid butt, without production of gas or H<sub>2</sub>S (as shown in Figure 38);
- an H<sub>2</sub>S-producing *Salmonella* may be used to control this reaction, which would appear as blackening of the medium in a positive reaction.

### Lysine iron agar (LIA)

Organisms that produce lysine decarboxylase in lysine iron agar (LIA) cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 40). H<sub>2</sub>S production is indicated by a blackening of the medium. Organisms lacking lysine decarboxylase (*e.g.*, *Shigella*) typically produce an alkaline slant (purple), an acid butt (yellow), no gas, and no H<sub>2</sub>S (see Table 13). *Proteus* and *Providencia* species will often produce a red slant caused by deamination of the lysine. LIA must be prepared so that the volume of medium in the tube is sufficient to give a deep butt. LIA tubes must have a deep butt because the decarboxylation reaction occurs only in anaerobic conditions.

- a) Prepare LIA medium according to manufacturer's instructions on the bottle.  
[**Note:** There are several commercially available formulations of dehydrated LIA. LIA may also be prepared from individual ingredients, but doing so may result in lot-to-lot variation.]
- b) Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant (*e.g.*, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes).
- c) Leave the screw-caps loose), and autoclave the medium.
- d) After autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is approximately 3.5-cm deep and the slant is approximately 2.5-cm long.
- e) Tighten the screw-top caps and store at 4°C for up to 6 months.

**Quality control:** For quality control of LIA, the following organisms should be adequate to confirm the biochemical response properties of the medium:

- *S. flexneri* should produce an alkaline slant and an acid butt without production of H<sub>2</sub>S;

- an H<sub>2</sub>S-producing *Salmonella* strain may be used to control the H<sub>2</sub>S reaction. *Salmonella* strains will most likely be lysine-positive and give an alkaline reaction in the butt of the tube.

### MacConkey agar (MAC)

MacConkey agar (MAC) is a differential plating medium recommended for use in the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. Colonies of *Shigella* on MAC appear as convex, colorless colonies about 2–3 mm in diameter. *S. dysenteriae* 1 colonies may be smaller. *S. Typhi* colonies are flat, colorless and usually 2–3 mm in diameter.

Several commercial brands of MAC are available. Most manufacturers prepare several formulations of MAC, which may vary in selectivity and thereby affect the isolation of *Shigella*. For example, some formulations of MAC do not contain crystal violet, a selective agent; these types are not as selective and should not be used for isolation of *Shigella*. Oxoid MacConkey Agar No. 3, Difco Bacto MacConkey Agar, and BBL MacConkey Agar are all suitable.

- Prepare MAC according to manufacturer's instructions. [**Note:** MAC can also be prepared from individual ingredients, but this produces more lot-to-lot variation than preparation of a commercially available dehydrated formulation.]
- Sterilize the medium by autoclaving at 121°C for 15 minutes.
- Cool to 50°C and pour into Petri plates (to a uniform depth of 3–4 mm).
- Leave lids ajar for about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 month. If plates are to be stored for more than a few days, put them in a sealed plastic bag to prevent drying.

**Quality control:** For quality control of MAC, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* should produce pink to red colonies with good to excellent growth; and,
- *S. flexneri* should produce colorless colonies with fair to good growth, but *S. dysenteriae* 1 colonies may be smaller.

### Martin-Lewis (ML) agar medium

(Refer to "Gonococcal Selective Media," listed earlier in this Appendix.)

### Modified Thayer-Martin (MTM) agar medium

(Refer to "Gonococcal Selective Media," listed earlier in this Appendix.)

## Motility medium

Because *Shigella* are always nonmotile, motility medium is a useful biochemical screening test. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39).

Nonmotile organisms do not grow out from the line of inoculation.

- a) Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. [**Note:** Several commercial dehydrated formulations of motility agar are available. This medium can also be prepared from individual ingredients, but this results in more lot-to-lot variation than commercial preparations.]
- b) Heat to boiling to make sure medium is completely dissolved.
- c) Dispense into tubes with screw-caps (or other types of containers), leaving the caps loose, and sterilize at 121°C for 15 minutes.
- d) Allow the medium to solidify upright, forming a deep butt with no slant (e.g., about 4–5 ml of medium per 13 x 100-mm screw-cap tube). When the medium is solidified and cooled, leave caps loose until the surface of the medium has dried.
- e) Tighten caps and store at 4°C for up to 6 months.

**Quality control:** For quality control of motility medium, the following organisms should be adequate:

- *E. coli* is motile;
- *Shigella* spp. are nonmotile.

The surface of the medium should be dry when used. If moisture has accumulated in the tube, carefully pour it out before the tube is inoculated. Moisture can cause a nonmotile organism to grow down the sides of the agar, creating a haze of growth and making it appear to be motile.

## Mueller-Hinton agar

Mueller-Hinton agar is the NCCLS-recommended medium used for standardized antimicrobial susceptibility testing of certain bacteria; the organisms in this document for which it is appropriate to use this formulation of Mueller-Hinton medium (i.e., unsupplemented Mueller-Hinton) are *S. Typhi*, *Shigella* spp., and *V. cholerae*.

[**Note:** Several formulations of Mueller-Hinton agar are commercially available. This laboratory manual suggests that Mueller-Hinton agar medium should not be prepared from individual ingredients because this can diminish the quality. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

- a) Follow manufacturer's instructions to prepare medium.
- b) After autoclaving, cool medium to 50°C in a water bath.
- c) Measure 60–70 ml of medium per plate into 15 x 150-mm plates, or measure 25–30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat-bottom glass or plastic Petri dishes on a level pouring surface to a **uniform depth of 3–4 mm**. Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.
- d) Freshly prepared plates may be used the same day or stored in a refrigerator (at 2°–8°C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35°–37°C) until the moisture evaporates (usually 10–30 min). **Do not leave lids ajar because the medium is easily contaminated.**

**Quality control:** Each new lot of Mueller-Hinton agar should be quality controlled before use by testing the *E. coli* ATCC 25922 standard strain for antimicrobial susceptibility testing. (This formulation of Mueller-Hinton agar can also be used for testing of gram-positive aerobes, in which case *S. aureus* ATCC 25923 can be used as a quality control strain.) **The pH of each new lot of Mueller-Hinton should be between 7.2 and 7.4; if the pH is outside this range, the pH of the medium should not be adjusted by the addition of acid or base, i.e., the batch of Mueller-Hinton plates should be discarded and a new batch of plates prepared.** If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory. Inhibition zone sizes / minimal inhibitory concentration (MIC) values for quality control are included in the antimicrobial susceptibility testing section of each pathogen-specific chapter.

### Mueller-Hinton agar plus 5% sheep (or horse) blood

Mueller-Hinton agar plus 5% sheep (or horse) blood is the NCCLS-recommended medium used for standardized antimicrobial susceptibility testing of certain bacteria; the organisms in this document for which it is appropriate to use this formulation of Mueller-Hinton medium supplemented with sheep (or horse) blood are *S. pneumoniae* and *N. meningitidis*.

[**Note:** Several commercial formulations of Mueller-Hinton agar are available. This medium should not be prepared from individual ingredients because this can diminish the quality and result in increased lot-to-lot variation. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

- a) Follow manufacturer's instructions to prepare medium.

- b) After autoclaving, cool medium to 50°C in a water bath.
- c) Add 5% sterile, defibrinated sheep (or horse) blood, *i.e.*, 50 ml of blood per liter of medium. (If a different volume of base medium is prepared, the amount of blood must be adjusted accordingly to 5%, *e.g.*, 25 ml of blood would be added to 500 ml of base medium.)
- d) Measure 60–70 ml of medium per plate into 15 x 150-mm plates, or measure 25–30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat-bottom glass or plastic Petri dishes on a level pouring surface to a uniform depth of 3–4 mm. Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.
- e) Freshly prepared plates may be used the same day or stored in a refrigerator (2°–8°C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35°–37°C) until the moisture evaporates (usually 10–30 minutes). **Do not leave lids ajar because the medium is easily contaminated.**

**Quality control:** Each new lot of Mueller-Hinton plus sheep blood agar (or horse blood, if preparing Mueller-Hinton for antimicrobial susceptibility testing of *S. pneumoniae* with trimethoprim-sulfamethoxazole [cotrimoxazole]) should be quality controlled before use by testing the *S. pneumoniae* standard strain (for quality control of *S. pneumoniae* and *N. meningitidis*). **The pH of each new lot of Mueller-Hinton should be between 7.2 and 7.4; if outside this range, the pH of the medium should not be adjusted by the addition of acid or base; the batch of plates should be discarded and a new batch of plates prepared.** If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory. Inhibition zone sizes and MIC values for quality control are included in the antimicrobial susceptibility testing section of each pathogen-chapter.

### Phosphate buffered saline (PBS)

The formula for this medium is:

Sodium dihydrogen phosphate	7.0 g
Disodium hydrogen phosphate	7.0 g
Distilled water	1000.0 ml

To prepare 0.1 M PBS, pH 7.2: Dissolve ingredients in distilled water. Adjust pH to 7.2 with 1 N acid or base. Dispense buffer in 500-ml bottles, and autoclave at 121°C for 15 minutes. Label bottles with the reagent name, date prepared, and the expiration date.

PBS has a shelf-life of one year if stored at room temperature (25°C).

## Physiological saline

(0.85% saline, also referred to as “Physiologic” saline or “Normal” saline)

Physiological saline is used in many different microbiological techniques. The formula for this saline is:

NaCl	8.5 g
Distilled water	1 liter

Dissolve the NaCl in water, heating if necessary. Physiological saline may be sterilized by autoclaving or membrane filtration. Store physiological saline at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

**Formalinized physiological saline** is physiological saline with the addition of formalin (formaldehyde). Follow the instructions above for preparation of physiological saline, and **after autoclaving** add 5 ml of 36% – 38% formaldehyde solution. Do not autoclave after the addition of formaldehyde to the saline.

## Polysaccharide medium

Polysaccharide medium is used to detect the production of polysaccharide from sucrose, and consists of TSA with 1% sucrose. The medium is included in this laboratory manual to assist in the identification of *N. gonorrhoeae*, which has a negative reaction. The formula for this medium is:

Tryptone soy agar (TSA)	40 grams
Distilled water (endotoxin-free; ETF)	1000.0 ml
Reagent-grade sucrose	(10% solution, preferably in distilled water)

**If reagent-grade sucrose is not available, white table sugar may be an acceptable substitute**, but brown sugar is not appropriate for preparation of this medium.

- Suspend TSA in the distilled, ETF water.
- Autoclave at 121°C for 15 minutes. Cool to 50°C.
- Prepare a 10% sucrose solution using the reagent-grade sucrose, and filter-sterilize it using a 0.45-micron filter.
- Aseptically add sucrose solution to agar to give a final concentration of 1% (wt/vol).
- Dispense 20–25 ml volumes in 100-mm Petri dishes.

Store medium refrigerated (at 4°–10°C) until used. Pre-warm the medium to room temperature prior to inoculation.

**Quality control:** For quality control of polysaccharide medium, the following organisms may be used:

- *N. gonorrhoeae* and *N. meningitidis* are two examples of organisms that do not produce polysaccharide from sucrose, and therefore exhibit a negative reaction (no color change) with the addition of Gram's iodine to the incubated inoculated medium.
- *N. polysaccharea* and *N. mucosa* are two examples of polysaccharide-positive organisms, and will exhibit a color change to dark brown to blue-black with the addition of Gram's iodine to the incubated inoculated medium.

### ***Salmonella-Shigella* agar (SS agar)**

SS agar is a highly selective medium for isolation of *Salmonella* and *Shigella*, although **it should not be used for isolation of *Shigella dysenteriae* type 1 because some strains are inhibited**. *S. Typhi*, which is lactose-negative, produces smooth, colorless, transparent or translucent colonies that may or may not have black centers indicating production of H<sub>2</sub>S. Lactose-positive colonies are pink surrounded by a zone of bile precipitation.

**Quality control:** To quality control SS agar, *Salmonella* should produce good growth of colorless colonies that may have black centers, whereas *E. coli* should grow poorly and appear as pink colonies.

### **Selenite broth (SEL)**

Selenite broth (SEL) is a frequently used as an enrichment broth for *Salmonella*, including *S. Typhi*. It may be advantageous for a laboratory to use SEL because it can also be used for enrichment for *Shigella*. SEL should only be incubated for 14–16 hours at 35°–37°C. After incubation, selenite broth should be streaked to selective agar (e.g., HE or XLD).

**Quality control:** After overnight enrichment in SEL, *Salmonella* spp. typically produce good to excellent growth when streaked on MacConkey agar.

### **Sulfide-indole-motility medium (SIM)**

Sulfide-indole-motility medium (SIM) is a commercially available combination medium that combines three tests in a single tube: hydrogen sulfide (H<sub>2</sub>S) production, indole production, and motility. The indole reaction is not useful for screening suspected *Shigella* isolates because strains vary in their reactions in this test. SIM is inoculated in the same way as motility agar (*i.e.*, by using a needle to stab about 1–2 cm down into the medium, and is incubated overnight at 35°–37°C). The motility reaction in SIM is read the same as for motility medium. As in Kligler iron agar or triple sugar iron agar, H<sub>2</sub>S production is indicated by blackening of the medium. Indole production can be tested by either the filter paper method or by adding Kovac's reagent to the tube.

- a) Follow manufacturer's instructions to weigh out and suspend dehydrated SIM medium.
- b) Heat to boiling to make sure the medium is completely dissolved.
- c) Dispense into tubes and sterilize by autoclaving for 15 minutes at 121°C.

**Quality control:** For quality control of SIM medium, the following organisms may be used:

- *E. coli* is indole positive, H<sub>2</sub>S-negative, and motility positive;
- an H<sub>2</sub>S-producing *Salmonella* strain may be used to control the H<sub>2</sub>S reaction and will most likely be motile and indole negative;
- *Shigella* are motility negative and H<sub>2</sub>S-negative but are variable for the indole reaction.

### Thiosulfate citrate bile salts sucrose agar (TCBS)

TCBS is a selective medium used to isolate *V. cholerae* from fecal specimens.

- a) Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. [**Note:** Several commercial brands of thiosulfate citrate bile salts sucrose agar (TCBS) agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.]
- b) Heat with agitation, until the medium is completely dissolved.
- c) Cool agar in a water-bath until cool enough to pour (50°–55°C).
- d) Pour into Petri plates, leaving lids ajar about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 week.

**Quality control:** Each new lot should be quality controlled before use because TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity.

- *V. cholerae* O1 should show good growth of yellow colonies; and,
- *E. coli* should have none to poor growth of translucent colonies.

### Todd-Hewitt broth

Todd-Hewitt broth is used (in the context of this laboratory manual) to incubate *S. pneumoniae* before re-testing when a Quellung reaction is not observed with growth in cell suspension from a blood agar plate. It is suggested that laboratories use a commercially available dehydrated formulation to prepare Todd-Hewitt broth, when possible.

- a) Prepare the Todd-Hewitt broth according to the instructions on the label of the dehydrated medium.

- b) Dispense 1 ml into 15 x 125-mm tubes, autoclave at 121°C for 20 minutes, cool, and store at 4°C.

**Quality control:** For quality control of Todd-Hewitt broth, inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae*; incubate overnight at 35°C; the broth should be turbid the next day. Subculture the broth onto a blood agar plate to test for proper growth characteristics of *S. pneumoniae*.

### Triple sugar iron agar (TSI)

(Refer to “Kligler iron agar and triple sugar iron agar,” listed earlier in this Appendix.)

### Tryptone-based soy agar (TSA)

TSA is a general-purpose tryptone-based agar medium (also commonly referred to as Trypticase soy agar or Tryptic soy agar) used with or without blood for isolating and cultivating a number of microorganisms. The medium should appear straw colored (*i.e.*, a yellowish to gold coloring). TSA is also used for determining the X- and V-factor requirements of *H. influenzae* (as is HIA).

- a) Prepare the TSA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks. The medium should be fully dissolved with no powder on the walls of the vessel before autoclaving; stirring over heat may help the powder dissolve more rapidly.
- b) Autoclave at 121°C for 20 minutes.
- c) Cool to 50°C and pour into 15x100-mm Petri dishes.
- d) Allow medium to solidify and condensation to evaporate before placing plates in plastic bags and storing at 4°C until they are used.

**Quality control:** Each freshly prepared or purchased batch of TSA should undergo quality control testing; follow instructions provided by the manufacturer.

- In general, *E. coli* is an organism that should show good growth on TSA.
- If a laboratory is using TSA to test suspect *H. influenzae* for growth factor requirements, it is suggested that the quality control include testing of a known *H. influenzae* isolate for X and V- factor requirements. To test the X and V requirements, inoculate a fresh plate of TSA with a control strain of *H. influenzae* (*e.g.*, ATCC 49247, which should be in stock for laboratories performing antimicrobial susceptibility testing); X, V, and XV disks should be placed on the inoculated plate identical to that shown in Figure 3. *H. influenzae* should grow only around the XV disk.

### Tryptone soy broth (TSB)

TSB (also commonly referred to as Trypticase soy broth or Tryptic soy broth) is used for making suspensions of *H. influenzae* prior to testing for X- and V- factor requirements. (HIA, sterile saline or phosphate-buffered saline [PBS] may be substituted for TSB when making the *H. influenzae* suspension for X and V factor testing.)

- a) Prepare the TSB according to the instructions on the label of the dehydrated medium.
- b) Dispense 5 ml into 15 x 125-mm tubes, autoclave at 121° C for 20 minutes, cool, and store at 4° C.

**Quality control:** Inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae*; incubate overnight at 35°C; the broth should be turbid the next day. Use a blood agar plate to subculture the broth to test for proper growth characteristics of *S. pneumoniae*. [**Note:** *H. influenzae* is not an appropriate organism for quality control of TSB because TSB lacks the X and V factors *H. influenzae* requires to grow.]

### Tryptone soy sheep blood agar with gentamicin

Sheep blood agar with gentamicin is used for the primary isolation of *S. pneumoniae* from nasopharyngeal swabs.

Prepare a stock solution of gentamicin by adding 80 mg of gentamicin in 32 ml of distilled water; the stock solution contains 2.5 mg of gentamicin/ml. Sterilize by filtration and dispense in 1.0-ml amounts, store at -20°C to -70°C.

To prepare sheep blood agar with gentamicin:

- a) Add 1 ml of the stock solution of gentamicin to 500 ml of molten agar (prepared according to manufacturer directions). Add the gentamicin at the same time the defibrinated sheep blood is added.
- b) Place the plates in plastic bags and store them at 4°C after they solidify. The agar should appear only slightly darker than the medium without gentamicin. If the agar is not bright red, discard and prepare a new batch.

**Quality control:** The quality control testing of freshly prepared or purchased gentamicin-blood medium is the same as for the (TSA-sheep) blood agar without gentamicin.

### Urea medium

Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color (Figure 40). Urease-negative organisms do not change the color

of the medium, which is a pale yellowish-pink. *Shigella* are always urease-negative (Table 15).

- a) Follow manufacturer's instructions for preparation of urea medium. [**Note:** Several commercial brands of urea medium are available, some of which require the preparation of a sterile broth that is added to an autoclaved agar base. Some manufacturers have sterile prepared urea concentrate available for purchase.] Prepare urea agar base as directed on the bottle.
- b) Sterilize at 121°C for 15 min.
- c) Cool to 50°–55°C, then add urea concentrate according to manufacturer's directions. (Before adding the urea to the agar base, make sure the agar base is cool since the urea is heat labile.)
- d) Mix and distribute in sterile tubes. Slant the medium during cooling so that a deep butt is formed.

**Quality control:** For quality control of urea medium, the following organisms are adequate:

- *Proteus* species produce urease;
- *E. coli* is urease negative.

### Xylose lysine desoxycholate agar (XLD)

Xylose lysine desoxycholate agar (XLD) is a selective differential medium suitable for isolation of *Shigella* and *Salmonella* from stool specimens. Differentiation of these two species from non-pathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulfide production. *Shigella* colonies on XLD agar are transparent pink or red smooth colonies 1–2-mm in diameter (Figure 86). *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species (Figure 85). Coliforms appear yellow (Figure 87). *Salmonella* colonies are usually red with black centers but can also be yellow with black centers.

- a) Prepare according to manufacturer's instructions. [**Note:** Several commercial brands of XLD agar are available. This medium can also be prepared from individual ingredients, but results exhibit more lot-to-lot variation than a commercially available dehydrated formulation.]
- b) Mix thoroughly.
- c) Heat with agitation just until the medium boils. Do not overheat; overheating when boiling XLD or allowing the medium to cool too long may cause the medium to precipitate.
- d) Cool flask under running water until just cool enough to pour; avoid cooling the medium too long.

- e) Pour the XLD into Petri plates, leaving the lids ajar for about 20 minutes so that the surface of the agar will dry.
- f) Plates can be stored at 4°C for up to a week.

**Quality control:** For quality control of XLD, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *S. flexneri* should produce fair to good growth of transparent pink or red smooth colonies that are 1–2 mm in diameter;
- *S. dysenteriae* 1 may produce very small transparent or red colonies;
- *E. coli* should produce poor to fair growth of yellow colonies.

## Transport and storage media

### Cary-Blair medium, Amies medium, and Stuarts medium

Prepare each of these transport media according to the manufacturer's instructions. [**Note:** Several dehydrated formulations of Cary-Blair are commercially available; some require the addition of calcium chloride and some do not.] These media can also be prepared from individual ingredients; however, it is very difficult to make a well quality-controlled batch and so this manual recommends purchasing them from a manufacturer.

When the Cary-Blair medium is prepared, it should be dispensed into containers in sufficient volume so that swabs will be covered by at least 4 cm of medium. For example, 5- to 6-ml amounts may be dispensed into 13 x 100-mm screw cap tubes. With the caps loosened, sterilize the medium by steaming (**not** by autoclave) at 100°C for 15 minutes. Tighten the caps after sterilization, and store the medium at 15°–30°C.

These media are quite stable if stored in tightly sealed containers in a cool dark place so that the medium does not dry out. Each may be used for up to 1 year as long as no loss of volume, visible contamination (e.g., foreign objects or bacterial growth), or color change is observed. Prepared Amies medium that has been stored for longer than 9 months, however, should be freshly steamed and the charcoal re-suspended before use.

### Dorset egg medium

Dorset egg medium (DE) is a good choice for the long-term survival of *S. pneumoniae* isolates (up to 44 days), *H. influenzae* isolates (up to 21 days) and *N. meningitidis* isolates (up to 21 days) at room temperature. The formula for this medium includes physiologic (normal) saline and whole hen's eggs.

- a) Combine sterile 0.85% (normal) saline solution with beaten whole hen's eggs in a 1:3 ratio.

- b) Inspissate (*i.e.*, thicken) the mixture in an electric inspissator at 80°C for 60 minutes.

### Greaves solution

Greaves solution can be used in the process of preparation of isolates for frozen storage, as described in Appendix XI of this manual. The formula for this medium is:

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

- a) Mix all ingredients and let them dissolve for 2–3 hours.
- b) Filter-sterilize the solution.
- c) Transfer the solution to a sterile tube.
- d) Incubate for 2 days at 35°–37°C (to control the sterility of the medium).
- If contamination is observed, discard the solution and prepare a new batch.
- e) Store at 4°C.

### JEMBEC® Plates

Jembec® plates are commercially available kits containing a CO<sub>2</sub>-generating system and a medium that will support the growth of gonococcus.

### Skim-milk tryptone glucose glycerol (STGG) transport medium

STGG medium is used for transport (and sometime storage) of nasopharyngeal secretions on swabs. The formula for this medium is:

Skim milk powder (from grocery or, <i>e.g.</i> , Difco)	2 g
TSB (from, <i>e.g.</i> , Oxoid)	3 g
Glucose	0.5 g
Glycerol	10 ml
Distilled water	100 ml

- a) Mix to dissolve all ingredients.
- b) Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials.
- c) Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds).
- d) Tighten caps after autoclaving.

- e) Store STGG frozen at -20°C or refrigerate until use.
- Use STGG medium within 6 months of preparation.

### Transgrow medium

Transgrow medium is a selective medium for the transport and isolation of *N. gonorrhoeae*. It should be prepared according to manufacturer's instructions. [Note: Transgrow medium is a chocolate agar plus three antibiotics and may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when the medium prepared from commercially available dehydrated preparations.]

### Trans-Isolate (T-I) Medium

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

When preparing T-I medium, use glass serum bottles with flange-type, slotted rubber plugs and aluminum crimp seals. Any size serum bottle that has at least a 20-ml capacity can be used, provided that the combined volume of solid and liquid phases equals approximately one-half the capacity of the bottle.

T-I medium includes solid and liquid phases; 0.1 M MOPS buffer (*i.e.*, 3-(N-morpholino) propanesulfonic acid buffer) with a pH of 7.2 is used in the preparation of both the solid and liquid phases of T-I medium. NaOH can be used to adjust the pH, and distilled water should be used to create the appropriate volume of 0.1 M solution (approximately 21 g MOPS: 1000 ml distilled water).

#### a) Solid phase:

Activated charcoal	2.0 g
Soluble starch	2.5 g
Agar agar ( <i>e.g.</i> , from Difco)	10.0 g
0.1 M MOPS buffer, pH 7.2	500 ml

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

## b) Liquid phase:

TSB	30.0 g
Gelatin (e.g., from Difco)	10.0 g
MOPS buffer [0.1 M, pH 7.2]	500.0 ml

1. Heat the medium to dissolve the gelatin and avoid coagulation.
2. Autoclave at 121°C for 15 minutes.
3. *Optional:* the addition of growth supplement (e.g., IsoVitaleX or Vitox) to the liquid phase of T-I medium can help support the growth of *H. influenzae*.
  - To add growth supplement to the entire batch of liquid phase medium, use aseptic technique to add a total of 10 ml of the growth supplement to the cooled liquid medium.
  - To add growth supplement to individual bottles, add 0.1 ml of the supplement to the liquid phase contained in an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.

## c) Addition of the liquid phase to the solid phase

1. Dispense 5 ml of the broth aseptically into each of the bottles containing the solid-phase slants.
2. Seal with sterile rubber stoppers and aluminum caps.
  - Use a hand-crimping tool to fasten the aluminum caps if an automated system is not available.

T-I bottles can be stored and used for at least 2 years if tightly capped and stored at 4°C. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature.

Before use, perform quality control of the T-I medium: check several uninoculated bottles for sterility at 35°C. Inoculate several bottles with *N. meningitidis* and check their ability to support meningococcal growth at 35°C.

Before inoculation, the bottles should be pre-warmed in the incubator (at 35°–37°C) or allowed to reach room temperature (i.e., 25°–30°C).

## Miscellaneous reagents

### Gram stain (Hucker Modification) reagents

The Gram stain (Hucker modification) requires the use of two stains (e.g., crystal violet and safranin or carbol-fuchsin), Gram's iodine, and a decolorizing agent (e.g., ethyl alcohol). Individual reagents and Gram stain kits are available commercially from several laboratory supply sources. Alternatively, follow the

methods for preparation of the individual reagents (as presented in steps a–d below).

- a) **Ammonium oxalate-crystal violet** contains two solutions (solution a and solution b).

*Solution a*

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

*Solution b*

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

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

- b) **Gram's iodine** must be protected from light.

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

1. Combine the crystalline iodine, potassium, and distilled water to prepare an iodine solution.
  - Grinding the dry chemicals in a mortar with small additions of distilled water may be helpful in preparing the iodine solution.
2. Store the Gram's iodine solution in a dark bottle and protected from light so it does not degrade.

- c) **Decolorizer** is commonly ethyl alcohol. (Some kits use acetone or an acetone-alcohol combination.)

95% ethyl alcohol

- d) **Counterstain** is commonly either safranin or carbol-fuchsin. Ziehl-Nielsen carbol-fuchsin is considered by many to be a more effective counter-stain than carbol-fuchsin.

1. **Safranin**

*Stock solution:*

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

*Working solution:*

Safranin stock solution	10.0 ml
Distilled water	90.0 ml

a. Prepare the stock safranin solution by combining the Safranin-O with the 95% ethyl alcohol.

b. Combine 10-ml of the stock solution with 90-ml of distilled water.

OR

**2. Ziehl-Nielsen carbol-fuchsin**

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

a. Dissolve fuchsin in alcohol.

b. Add the 5% phenol solution.

c. Let stand overnight.

d. Filter through coarse filter paper.

- This solution can be used as described or diluted 1:10.

When limited by resource availability, counterstain can be prepared as a 0.3–0.5 aqueous solution of the basic fuchsin.

### Loeffler's methylene blue stain

Loeffler's methylene blue provides a simple staining method for visualize the shape of bacterial cells; it does not determine whether bacteria are gram-positive or gram-negative. If determining whether an organism is gram-positive or gram-negative is essential, smears must be stained by Gram's method (*i.e.*, using reagents as described in "Gram stain", earlier in this Appendix). Because of the characteristic shape and arrangement of cells in *Neisseria* species, the methylene blue stain may provide an inexpensive, rapid method for detecting diplococci. (This laboratory manual recommends use of Loeffler's methylene blue stain in place of Gram stain for staining of suspect *N. gonorrhoeae* from specimens or cultures of non-sterile sites, but not for staining of *N. meningitidis* from sterile-site specimens.)

To prepare the Loeffler's methylene blue stain, add the components in the order presented in the following two steps to first prepare saturated ethanolic methylene blue and then the staining solution.

**a) Saturated ethanolic methylene blue**

Methylene blue powder	1.0 g
Ethanol (95%)	100 ml

### b) Staining solution

KOH (1% aqueous solution)	1 ml
Distilled water	99 ml
Ethanolc methylene blue solution ( <i>step 1</i> )	30 ml

Loeffler's methylene blue reagent must be 'ripened by oxidation,' and the ripened reagent is called polychrome methylene blue. Normally, oxidation takes several months, but it can be hastened by aerating the reagent: Place the reagent in bottles filled no more than half-full and shake the bottle frequently.

Loeffler's methylene blue stain improves with age, and the shelf-life of this reagent is 5–10 years; thus, the reagent can be prepared in batches large enough to last for this time period.

### Nitrate reduction test reagents

These media and reagents are used to perform the nitrate reduction test for the confirmation of an isolate as *N. gonorrhoeae*. The test is performed in a nitrate broth composed of heart infusion broth containing 0.2% potassium nitrate.

The formula for the nitrate reduction test medium is:

Heart infusion broth	25.0 g
Potassium nitrate	2.0 g
Distilled water	1000.0 ml

Reagents for developing the nitrate reduction tests are as follows:

#### Nitrate Reagent A (Sulfanilic acid solution): 0.8% in 5 N acetic acid\*

4-aminobenzene sulfonic acid	0.5 g
Acetic acid, glacial	20 ml
Distilled water	100 ml

- 1) Dissolve 0.5 g of 4-aminobenzene sulfonic acid in 30 ml of glacial acetic acid.
- 2) Add 100 ml of distilled water and filter.

Store Nitrate Reagent A at room temperature (15°–30°C) in the dark. Reagents may be stored in dark brown glass bottles or in clear bottles wrapped in aluminum foil to ensure darkness. **Nitrate Reagent A is stable for one month.**

#### Nitrate Reagent B (alpha-naphthylamine solution): 0.6% in 5 N acetic acid\*

N,N-dimethyl-1 naphthylamine	0.1 g
Distilled water, boiling	100 ml
Acetic acid, glacial	30 ml

- 1) Dissolve 0.1 g of N,N-dimethyl-1 naphthylamine in 100 ml of boiling distilled water. Cool to room temperature.

2) Add 30 ml of glacial acetic acid.

3) Filter.

Store Nitrate Reagent B at room temperature (15°–30°C). Reagents may be stored in dark brown glass bottles or in clear bottles wrapped in aluminum foil to ensure darkness. **Nitrate Reagent B is stable for up to one week (7 days).**

**Zinc powder:** reagent grade. Store at room temperature.

\* **Warning:** 5 Normal (glacial) acetic acid is corrosive. Contact with skin may cause blisters and burns. In case of contact, flush eyes and skin immediately with plenty of water for at least 15 minutes.

### Nitrocefin reagent for $\beta$ -lactamase (penicillinase) test

The nitrocefin test is used to detect  $\beta$ -lactamase. Reagents should be warmed to room temperature prior to use. There are two formulations of the liquid reagent for the nitrocefin test: one has a nitrocefin powder concentration of 500- $\mu$ g/ml, and the other has a nitrocefin powder concentration of 25- $\mu$ g/ml. The reagent used for the plate test contains 500  $\mu$ g of nitrocefin powder/ml and is dropped directly onto colonies on culture medium; in contrast, the reagent used for the tube test (in which bacterial cells are suspended in the reagent) contains only 25  $\mu$ g of nitrocefin/ml. Nitrocefin disks are also commercially available.

Because the nitrocefin reagent is expensive, this laboratory manual suggests that a commercially available nitrocefin disk be used because it is a more cost-effective means of performing the test than use of the liquid reagent (unless a laboratory is conducting surveillance for penicillin resistance in *N. gonorrhoeae* and will be performing the nitrocefin test on large numbers of isolates). If, however, a laboratory wants to prepare its own liquid nitrocefin reagent, instructions are below. (Methods for performing the nitrocefin test with liquid reagent are included in the *N. gonorrhoeae* chapter of this manual.) Because the reagent used for the tube method is more dilute than that used for the plate test, performing the nitrocefin test by the tube method using the liquid reagent is more cost-effective than the plate or disk method for testing large numbers of isolates.

Note that preparation of the nitrocefin solution requires dimethyl sulfoxide (DMSO;  $\text{CH}_2\text{SO}_4$ ), and because of the hazardous nature of DMSO some suppliers may require a letter of justification for its purchase.

Materials for preparation of nitrocefin solution include:

Nitrocefin powder	(0.5 g for stock solution of 100 ml)
0.1 M phosphate buffer, pH 7.2	(100 ml for stock solution; 1:20 dilution for tube test)

DMSO (dimethyl sulfoxide)  
Graduated cylinder (50 ml)  
Screw-cap/snap-capped tubes (5 ml capacity)  
Pasteur pipets (sterile)

**Nitrocefin solution for plate test** (“stock solution”; 500 µg/ml)

- a) Weigh 0.5 g nitrocefin powder in a weigh boat or beaker. Transfer nitrocefin powder to graduated cylinder.
- b) Using a sterile glass pipette, add a few drops of DMSO to the nitrocefin powder. Swirl until the powder dissolves.
- c) Make volume up to 100 ml with 0.1 M phosphate buffer, pH 7.2.
- d) Dispense nitrocefin reagent in 5 ml volume into screw-cap/snap-capped tubes.
- e) Label tubes with the following information: Reagent name, date prepared, the expiration date when moved to storage at 4°–10°C, and hazard code for DMSO. (*This information should also be logged in the QC log.*)

**Nitrocefin solution for tube test** (25 µg/ml) (*Growth suspended in nitrocefin reagent contained in tubes / microtiter plate wells*)

- a) Prepare the stock nitrocefin solution (500 µg nitrocefin/ml) as described in the “Plate test nitrocefin solution, stock solution” section above.
- b) Dilute the stock solution 1:20 with 0.1 M phosphate buffer, pH 7.2.
- c) Dispense 3-ml volumes of the diluted nitrocefin solution into screw-cap or snap-capped tubes.
- d) Label tubes with the following information: reagent name, date prepared, the expiration date, and hazard code for DMSO. (*This information should also be logged in the work record.*)

Nitrocefin reagents may be prepared in bulk, dispensed in small aliquots (1–2 ml), and stored at -20°C or -70°C indefinitely if no color change (from colorless/yellow to pink) is observed. If a tube of the reagent is ‘in-use,’ the reagent may be stored for up to one year at 4°–10°C if no color change is observed.

**Quality control:** Perform quality control with each newly prepared batch of nitrocefin reagent or each newly purchased batch of nitrocefin disks.

- An example of a β-lactamase negative control strain is *N. gonorrhoeae* ATCC 49226.
- Examples of β-lactamase positive control strain are *H. influenzae* ATCC 49247 and *N. gonorrhoeae* P681E (available from the CDC’s Gonorrhea Research Laboratory, see Appendix 14).

### Oxidase reagent (Kovac's oxidase)

Kovac's oxidase reagent is used to test for the presence of cytochrome oxidase; *N. gonorrhoeae*, *N. meningitidis*, and *V. cholerae* are all oxidase-positive and exhibit a purple reaction when exposed to this reagent. The formula for Kovac's oxidase follows:

<i>N,N,N',N'</i> -Tetramethyl- <i>p</i> -phenylenediamine dihydrochloride	0.05 g
Distilled water	5.0 ml

Dissolve the reagent in purified water. (Do not heat to dissolve.)

### Preparation of 1% Kovac's oxidase reagent from powder

To prevent deterioration of stock oxidase-reagent powder, store in a tightly closed bottle in a desiccator kept in a cool dark area. Prepare 10 ml of a 1.0% tetramethyl-*p*-phenylenediamine hydrochloride solution in distilled water. Dispense the reagent in 1-ml aliquots and store frozen at -20°C.

For use, thaw a 1-ml vial and either use the liquid reagent to moisten filter paper or a swab or prepare dried strips of filter paper.

- To prepare dried treated filter paper, immediately after the vial is thawed, wet as many strips of filter paper as possible on a nonporous surface (*i.e.*, Petri dish, glass plate). Let the strips dry in air or in the incubator. When the strips are completely dry, place them in a tightly capped tube/bottle and refrigerate at 4°C. The strips can then be used as needed.

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

Instead of Kovac's oxidase reagent (described above), some laboratories may use Gordon and McLeod's reagent. Gordon and McLeod's reagent is prepared to a 1% solution (as is Kovac's oxidase), but instead of the tetramethyl- reagent used for Kovac's reagent, Gordon and McLeod's reagent uses dimethyl-*p*-phenylenediamine dihydrochloride. Gordon and McLeod's oxidase is a more stable reagent but oxidase reactions take up to 30 minutes to occur, instead of 5 minutes; it should also be noted that oxidase-positive reactions with Gordon and McLeod's reagent are blue (not purple). This laboratory manual suggests using Kovac's oxidase reagent if it is available.

**Quality control:** Positive and negative controls should be tested every time the reagent is prepared.

- *V. cholerae*, *N. meningitidis*, and *N. gonorrhoeae* are oxidase-positive
- *E. coli* and *S. pneumoniae* are oxidase negative.

### Sodium desoxycholate reagent (0.5%) for string test

The string test is used to help identify *V. cholerae*. The formula for this reagent follows:

Sodium desoxycholate (also seen as “deoxycholate”)	0.5 g
Sterile distilled water	100.0 ml

Add sterile distilled water to sodium desoxycholate and mix well. Store at room temperature for up to 6 months.

**Quality control:** Each new batch of sodium desoxycholate should be quality controlled before use.

- Use a *V. cholerae* O1 strain as a positive control.
- *E. coli* may be used as a negative control.

## Preparation of turbidity standards

### Turbidity standards (McFarland turbidity standards)

Commercially prepared 0.5 McFarland turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland turbidity standard may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) solution to 99.5 ml of 1% (vol/vol) sulfuric acid ( $\text{H}_2\text{SO}_4$ ). The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland turbidity standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland turbidity standards may be stored for up to 6 months in the dark at room temperature (*i.e.*, 22°–25°C); discard after 6 months or sooner if any volume is lost. (Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred; if it has, a fresh turbidity standard should be prepared.) Before each use, shake the tube containing the turbidity standard well, so that the fine white precipitate of barium sulfate is mixed in the tube.

The composition of McFarland turbidity standards and the corresponding densities of bacteria (/ml) are presented in Table 23.

The accuracy of the density of a prepared McFarland turbidity standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland turbidity standard, the absorbance at a wavelength of 625 nm should be 0.08–0.1. Alternately, the accuracy of the McFarland turbidity standard may be verified by adjusting a suspension of a control strain (*e.g.*, *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts of colonies (Figure 50). The adjusted suspension should give a count of 108 colony forming units/ml. Figures 51 and 52 are helpful guides for how to read and compare the McFarland turbidity standard to a newly prepared cell suspension.

FIGURE 50: Flowchart procedure for preparation and quality control of the 0.5 McFarland turbidity standard

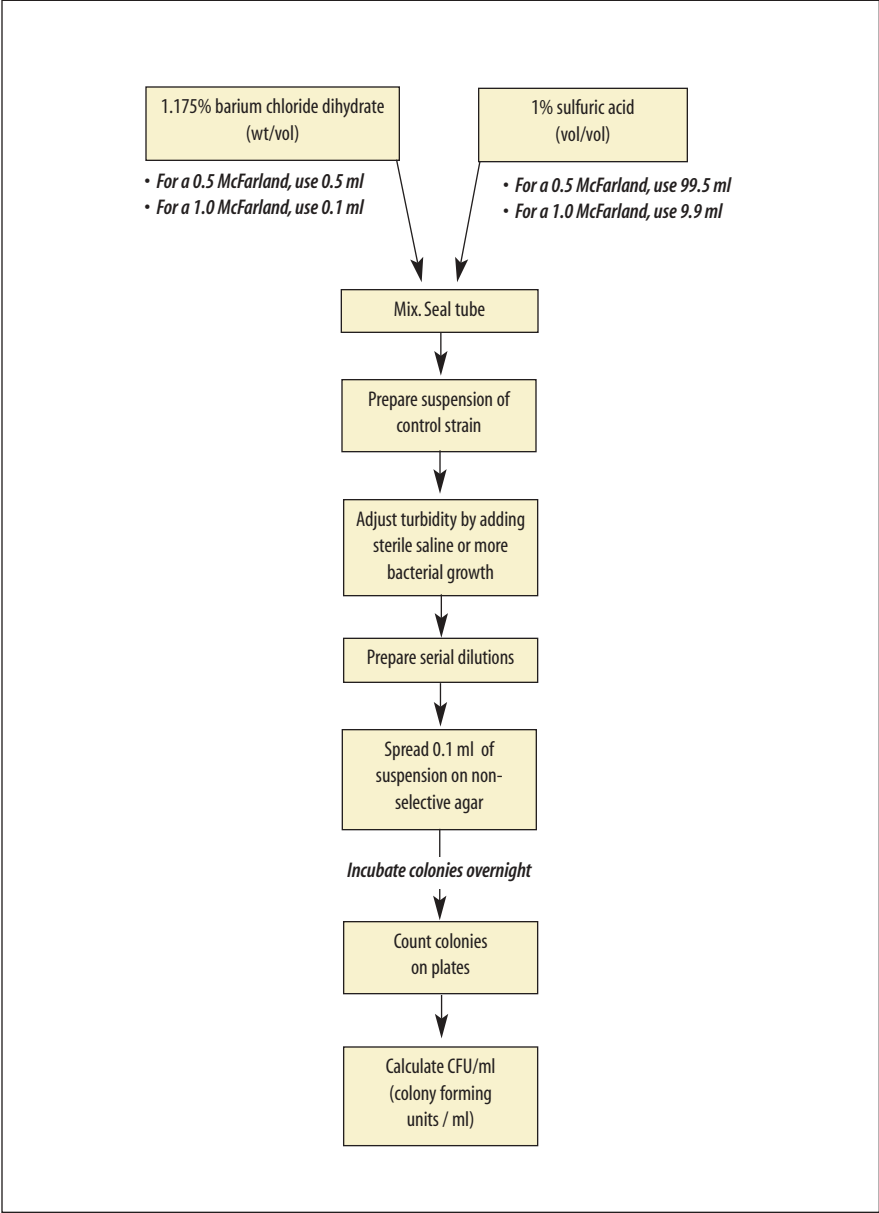


TABLE 23: Composition of McFarland turbidity standards

Turbidity standard number	Barium chloride dihydrate (1.175%)	Sulfuric acid (1%)	Corresponding approximate density of bacteria
0.5	0.5 ml	99.5 ml	$1 \times 10^8$
1	0.1 ml	9.9 ml	$3 \times 10^8$
2	0.2 ml	9.8 ml	$6 \times 10^8$
3	0.3 ml	9.7 ml	$9 \times 10^8$
4	0.4 ml	9.6 ml	$12 \times 10^8$
5	0.5 ml	9.5 ml	$15 \times 10^8$
6	0.6 ml	9.4 ml	$18 \times 10^8$
7	0.7 ml	9.3 ml	$21 \times 10^8$
8	0.8 ml	9.2 ml	$24 \times 10^8$
9	0.9 ml	9.1 ml	$27 \times 10^8$
10	1.0 ml	9.0 ml	$30 \times 10^8$

FIGURE 51: Comparison of the 0.5 McFarland turbidity standard with inoculum suspension



FIGURE 52: Background lines for viewing turbidity of a suspension in comparison to a turbidity standard



### Plate count method for testing a 0.5 McFarland turbidity standard

The goal of this procedure is to determine the number of bacteria per ml of fluid. A bacterial suspension equivalent in turbidity to a 0.5 McFarland turbidity standard contains approximately  $10^8$  bacteria per ml.

- 1) Prepare 0.5 McFarland turbidity standard, as described above.
- 2) Prepare a suspension of a test organism (*e.g.*, *E. coli* ATCC 25922) to match the density of the McFarland turbidity standard.
- 3) Make serial, 10-fold dilutions of the bacterial suspension in a suitable broth medium. (Examples of suitable broth media include Mueller Hinton broth, TSB, or PBS.) The following steps *a – i* describe the procedure for making the serial dilutions.

Materials necessary for testing of the 0.5 McFarland turbidity standard include: seven sterile screw-capped tubes, seven agar plates (with medium to support growth of the organism you're testing), and pipettes capable of measuring out 4.5 ml and 0.5 ml, respectively. In addition, a vortex machine is useful for vigorous mixing in tubes.

- a) Make sure you have seven screw-capped tubes, each capable of holding at least 10 ml of fluid. Prepare dilution tubes by adding 4.5 ml of sterile broth to each of the seven 10-ml tubes.
- b) Label the tubes from 1 to 7, indicating the dilution the tube will hold. Also label agar plates of the appropriate medium from 1 to 7.
- c) Add 0.5 ml of the bacterial suspension made up to 0.5 McFarland turbidity standard to the tube labeled 7 and mix vigorously.

- d) **Using the same pipette as in step C**, draw up and release the suspension several times into the pipette and then transfer 0.5 ml from tube 7 to tube 6 and mix vigorously.
- e) Continue this process of transferring 0.5 ml to each successive tube, **using the same pipette**, until you have completed the dilutions with tube 1. After vigorously mixing tube 1, use the pipette to draw up and release the suspension in the tube several times.
- f) Using the same pipette, transfer 0.1 ml from tube 1 to the plate labeled 1.
- g) Using the same pipette, transfer 0.1 ml from tube 2 to the plate labeled 2. Continue this process for tube 3 to plate 3, and tube 4 to plate 4. (If laboratorians unfamiliar with making bacterial suspensions to match a McFarland turbidity standard are responsible for the procedure, the process may be continued through tube 7. However, plating out the tubes with a higher concentrations of medium is not mandatory, because doing so would result in too many colonies to count when they grow.)
- h) Using a bent rod and starting with plate 1, spread the fluid on each plate over the entire surface of the plate. A bent rod can be made by using heat to bend a 2–5 mm diameter glass rod to an approximately 60°-angle, with the short end measuring approximately 5 cm. A bent, stainless steel metal rod of similar size can be used as an alternative to a glass rod. (The fluid can also be spread with a wire inoculating loop or needle bent to a 60°-angle, but spreading the fluid evenly is more difficult using these methods.)
- i) Incubate the plates overnight and count the number of colonies on each plate. It may be difficult to count the colonies on plates 4 through 7, and if there are more than 300 colonies per plate it should not be counted.

### Interpretation of plate count results

A 0.5 McFarland turbidity standard is equivalent to approximately  $10^8$  bacteria per ml. The original bacterial suspension that resembles the 0.5 McFarland turbidity standard could have a range of  $1.0 \times 10^8$  bacteria/ml to  $9.0 \times 10^8$  bacteria/ml. Within this range, the 0.5 turbidity standard is accurate; the difference will be evident in the number of bacteria that grow out on the plates.

After 0.5 ml of the original bacterial suspension (*i.e.*, which is equivalent to the 0.5 McFarland turbidity standard) is added to the 4.5 ml of broth in tube 7, a suspension of bacteria is produced that contains approximately  $10^7$  bacteria per ml. Then 0.1 ml of this suspension has been transferred to the plate marked 7, which translates to approximately  $10^6$  (1,000,000–9,000,000) bacteria present on that plate. If the bacteria were diluted correctly: approximately  $10^5$  (or 100,000 –

<sup>34</sup> All lists are limited and incomplete. Note that inclusion of a company or specific product does not imply endorsement by CDC or WHO.

900,000) bacteria should be present on the plate labeled 6; approximately 10,000–90,000 bacteria on the plate labeled 5; approximately 1,000–9,000 bacteria present on plate 4; approximately 100 to 900 bacteria present on plate 3; approximately 10 to 90 bacteria on plate 2; and, approximately 1 to 9 bacterial colonies would be present on plate 1. Each plate should have one-tenth the bacteria as the plate with the next higher number. Generally, the plate labeled 3 will be the plate that is counted; however, if there are more than 300 colonies present on plate 3, then plate 2 should be counted.

## Sources of prepared media and reagents

Although commercially prepared media and reagents are more expensive than media or reagents that can be prepared locally, commercially available items can be used (and may be preferable) in certain situations. Dehydrated media, for example, are often preferable to media prepared from individual components because of reduced lot-to-lot variation. It may also be desirable to purchase the supply of media and reagents to perform short-term studies rather than attempt formulation. The following media and reagents are available in most parts of the world from suppliers including, but **not limited to**: BBL (available from Becton, Dickinson and Company), bioMérieux, Difco (available from Becton, Dickinson and Company), Merck, Oxoid, and Quélab (Table 24); a partial listing of manufacturers, suppliers, and distributors with contact information is included in Appendix 13.<sup>35</sup> (The listing of supplies, media and reagents in this laboratory manual is not exhaustive, and availability of products from specific companies or suppliers may change. Inclusion of a company or product does not imply endorsement by CDC or WHO.) It is essential that each lot of materials has a satisfactory expiration date and that the date of expiration and lot number for commercial media are recorded in the laboratory.

In addition to media and reagents, laboratories must maintain their supplies (e.g., glassware) and equipment; Developing Health Technology is a company that provides low-cost laboratory equipment for developing countries, nongovernmental organizations (NGOs) and aid agencies. Furthermore, as noted elsewhere in this document, the manufacturer of the Etest® (AB Biodisk) may make materials available at a reduced price to laboratories in developing country settings. Contact information for these companies is available in Appendix 13.

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<sup>35</sup> All lists are limited and incomplete. Note that inclusion of a company or specific product does not imply endorsement by CDC or WHO.

TABLE 24: Partial listing of materials and suppliers / manufacturers

Description of item <sup>a</sup> <i><sup>a</sup> This is not intended to be comprehensive catalog of materials and suppliers.</i>	Sample listing of manufacturers <sup>b</sup> <i><sup>b</sup> Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.</i>
Acetone-alcohol (decolorizing agent)	Remel; Difco (BD); BBL (BD)
Agar agar	Remel; Oxoid; BBL (BD)
Ampicillin disks	Remel; Oxoid; BBL (BD)
Azithromycin disks	Remel; Oxoid; BBL (BD)
Bile salts	Quélab; Remel; Oxoid; BBL (BD)
Bismuth sulfite (BS) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Blood culture medium - <i>prepared</i>	bioMérieux; Oxoid
Bovine hematin	Quélab; BBL (BD)
Brain-heart infusion (BHI)	Quélab; Difco (BD); Oxoid; BBL (BD); Remel
Cary-Blair medium	Quélab; Difco (BD); Oxoid; BBL (BD)
Cefixime disks	Remel; Oxoid; BBL (BD)
Ceftriaxone disks	Remel; Oxoid; BBL (BD)
Chloramphenicol disks	Remel; Oxoid; BBL (BD)
Chocolate blood agar - <i>prepared</i>	bioMérieux; Remel; BBL (BD)
Chocolate blood agar + bacitracin - <i>prepared</i>	Remel; BBL (BD)
Ciprofloxacin disks	Remel; Oxoid; BBL (BD)
CO <sub>2</sub> -generating systems	Oxoid; Remel
Colistin disks	Quélab; Remel; Oxoid; BBL (BD)
Crystal violet	Quélab; Difco (BD); Remel; BBL (BD)
Cystine trypticase agar (CTA)	Quélab; Difco (BD); BBL (BD)
Defined growth supplement (e.g., IsoVitalX, Vitox, VX Supplement)	BBL (BD); Oxoid; Difco (BD)
Desoxycholate	Quélab; Remel
Desoxycholate citrate agar (DCA)	Quélab; Difco (BD); BBL (BD)
Etest® antimicrobial gradient strips	AB Biodisk; Remel; Fisher Scientific
Ethanol	Remel; Merck
Formalin (formaldehyde)	Remel; Merck
Furazolidone disks	Remel; Oxoid; BBL (BD)
Gonochek II® (enzyme substrate test)	TCS Microbiology
Gonococcus (GC) agar base medium	Quélab; Difco (BD); Oxoid; BBL (BD)
Gram stain kit	bioMérieux; Difco (BD); Remel; BBL (BD)
Gram-negative broth	Difco (BD); BBL (BD); Renek
Gram's iodine	Quélab; Difco (BD); Remel; BBL (BD)
<i>H. influenzae</i> serotyping antisera	Difco (BD); Remel

continued

TABLE 24: Partial listing of materials and suppliers / manufacturers, *continued*

Description of item <sup>a</sup> <sup>a</sup> This is not intended to be comprehensive catalog of materials and suppliers.	Sample listing of manufacturers <sup>b</sup> <sup>b</sup> Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.
<i>Haemophilus</i> test medium (HTM)	Oxoid; BBL (BD)
Heart infusion agar / broth	Quélab; Difco (BD); BBL (BD)
Hektoen enteric (HE) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Hemoglobin powder	Quélab; Difco (BD); Oxoid; BBL (BD)
Horse blood	Difco (BD); Oxoid
Jembec® plates	BBL (BD); Quélab
Kligler iron agar (KIA)	Quélab; Difco (BD); Oxoid; BBL (BD)
Kovac's oxidase reagent ( <i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine dihydrochloride)	Quélab; Merck
Lysine iron agar (LIA)	Quélab; Difco (BD); Oxoid; BBL (BD)
MacConkey agar (MAC)	Quélab; Difco (BD); Oxoid; BBL (BD)
Martin-Lewis medium - <i>prepared</i>	Quélab; BBL (BD)
Methylene blue	Quélab; Remel
Modified Thayer-Martin medium - <i>prepared</i>	Quélab; Remel; BBL (BD)
Motility medium	Quélab; Difco (BD); BBL (BD)
Mueller-Hinton agar - <i>prepared</i>	bioMérieux; Remel; BBL (BD)
Mueller-Hinton agar plus 5% sheep blood - <i>prepared</i>	bioMérieux; Remel; BBL (BD)
Mueller-Hinton agar/broth	Quélab; Difco (BD); Oxoid; Remel
<i>N. meningitidis</i> serogrouping antisera	Difco (BD); Remel
Nalidixic acid disks	Oxoid; BBL (BD)
Nicotinamide adenine dinucleotide (NAD; V factor)	Quélab; Merck
Nitrate broth	Quélab; Difco (BD)
Nitrate Reagents A and B	Remel; BBL (BD)
Nitrocefin (beta-lactamase)	Remel; Difco (BD); Oxoid; BBL (BD)
Optochin (p-disks)	Oxoid; BBL (BD)
Oxacillin disks	Oxoid; BBL (BD)
Penicillin disks	Oxoid; BBL (BD)
Peptone	Difco (BD); Oxoid
Permeable membrane screw-caps (for short-term storage of <i>N. meningitidis</i> at 4 °C)	(Biomedical Polymers, Inc., via) Fisher Scientific; VWR International
Phosphate buffered saline (PBS)	Quélab; Oxoid
Quad ID plate	Quélab; BBL (BD)
Quality control strains (type cultures)	American Type Culture Collection (ATCC); National Culture Type Collection (NCTC)
<i>S. pneumoniae</i> serotyping antisera	Omniserum; Remel; Difco (BD)

*continued*

TABLE 24: Partial listing of materials and suppliers / manufacturers, *continued*

Description of item <sup>a</sup> <sup>a</sup> This is not intended to be comprehensive catalog of materials and suppliers.	Sample listing of manufacturers <sup>b</sup> <sup>b</sup> Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.
Safranin	Quélab; Difco (BD); Remel; BBL (BD)
<i>Salmonella</i> (ser. Typhi) serotyping antisera	Remel; Difco (BD)
<i>Salmonella-Shigella</i> (SS) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Selenite broth (SEL)	Quélab; Difco (BD); Oxoid; BBL (BD)
Sheep blood	Remel; Quélab
Sheep blood agar (TSA + 5% sheep blood) - <i>prepared</i>	bioMérieux; BBL (BD)
Sheep blood agar + gentamicin - <i>prepared</i>	Quélab; BBL (BD)
<i>Shigella</i> antisera	Remel; Difco (BD)
Silica gel packets (for transport and short-term storage of some pathogens)	Scientific Device Laboratory, Inc.
Skim milk powder	Quélab; Difco (BD); BBL (BD)
Sodium polyanethol sulfonate (SPS)	Quélab; Oxoid
Spectinomycin disks	Oxoid; BBL (BD)
Sucrose (reagent-grade)	Quélab; BBL (BD)
Sulfide-indole-motility medium (SIM)	Remel; Oxoid; BBL (BD)
Superoxol reagent (30% H <sub>2</sub> O <sub>2</sub> )	Quélab; Merck
Tetracycline disks	Remel; Oxoid; BBL (BD)
Thiosulfate citrate bile salts sucrose agar (TCBS)	Quélab; Oxoid; BBL (BD)
Todd-Hewitt broth	Difco (BD); Oxoid; BBL (BD)
Trimethoprim-sulfamethoxazole (cotrimoxazole) disks	Remel; Oxoid; BBL (BD)
Triple sugar iron agar (TSI)	Quélab; Difco (BD); BBL (BD)
Tryptone (Trypticase) soy agar/broth	Quélab; Difco (BD); Oxoid; BBL (BD); bioMérieux
Tryptone (Trypticase) soy agar - <i>prepared</i>	BBL (BD); bioMérieux
Urea medium	Quélab; Difco (BD); Oxoid; BBL (BD)
<i>V. cholerae</i> antisera	Remel; Difco (BD)
VCA(T) supplement (to prepare Martin-Lewis medium)	Quélab; Oxoid; BBL (BD)
VCN(T) supplement (to prepare Modified Thayer-Martin)	Quélab; Oxoid; BBL (BD)
V-factor disks (NAD)	Remel; Oxoid
X-factor disks (haemin)	Remel; Oxoid; Quélab
XV-factor disks	Remel; Oxoid; Quélab
Xylose lysine desoxycholate agar (XLD)	Quélab; Difco (BD); Oxoid; BBL (BD); Remel
Zinc dust	Quélab; BBL (BD)



## 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 recap 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.<sup>36</sup> 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.

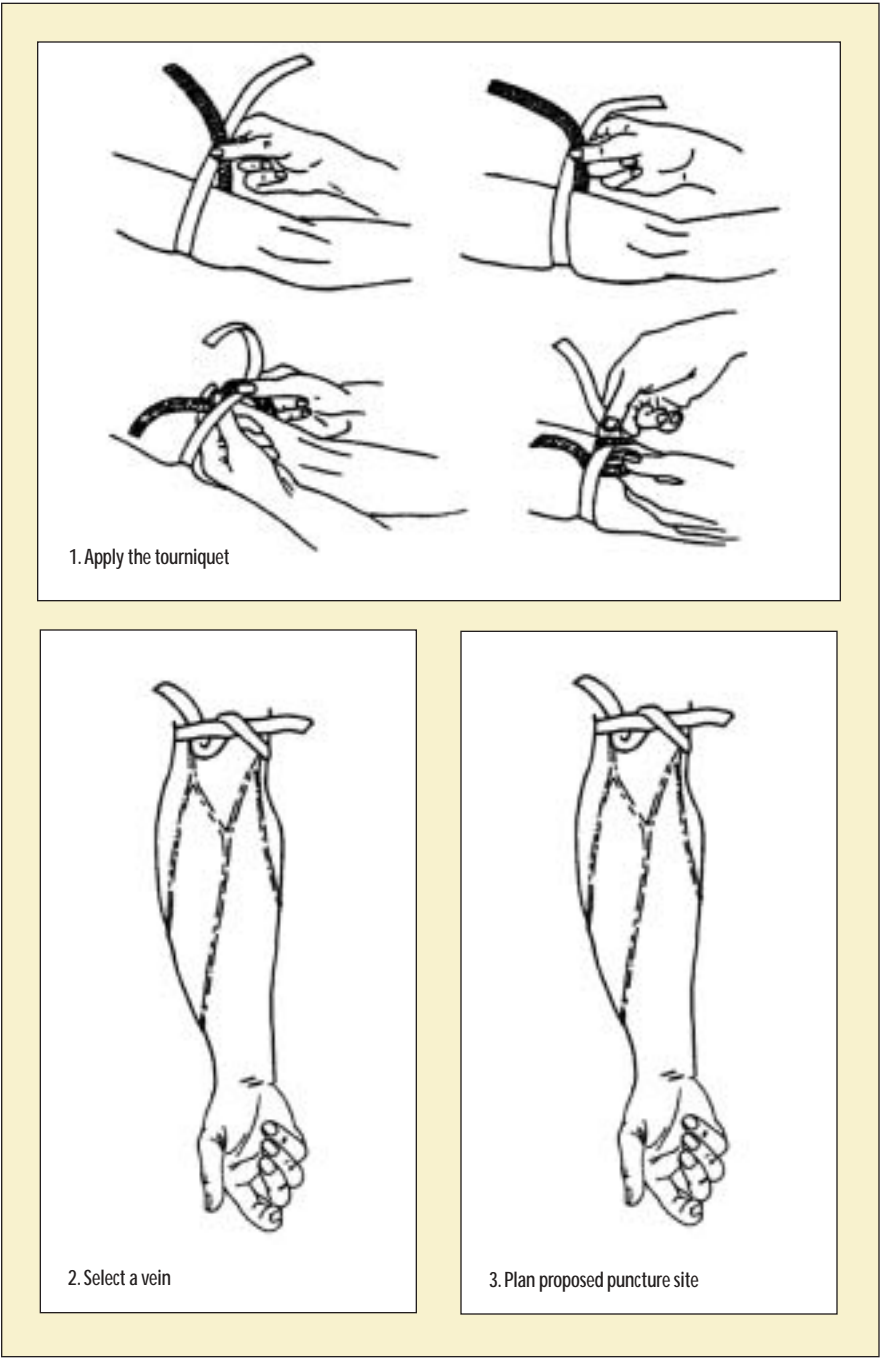
## Transport of blood specimens

**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.

---

<sup>36</sup> 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).<sup>37</sup>

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

<sup>37</sup> 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:

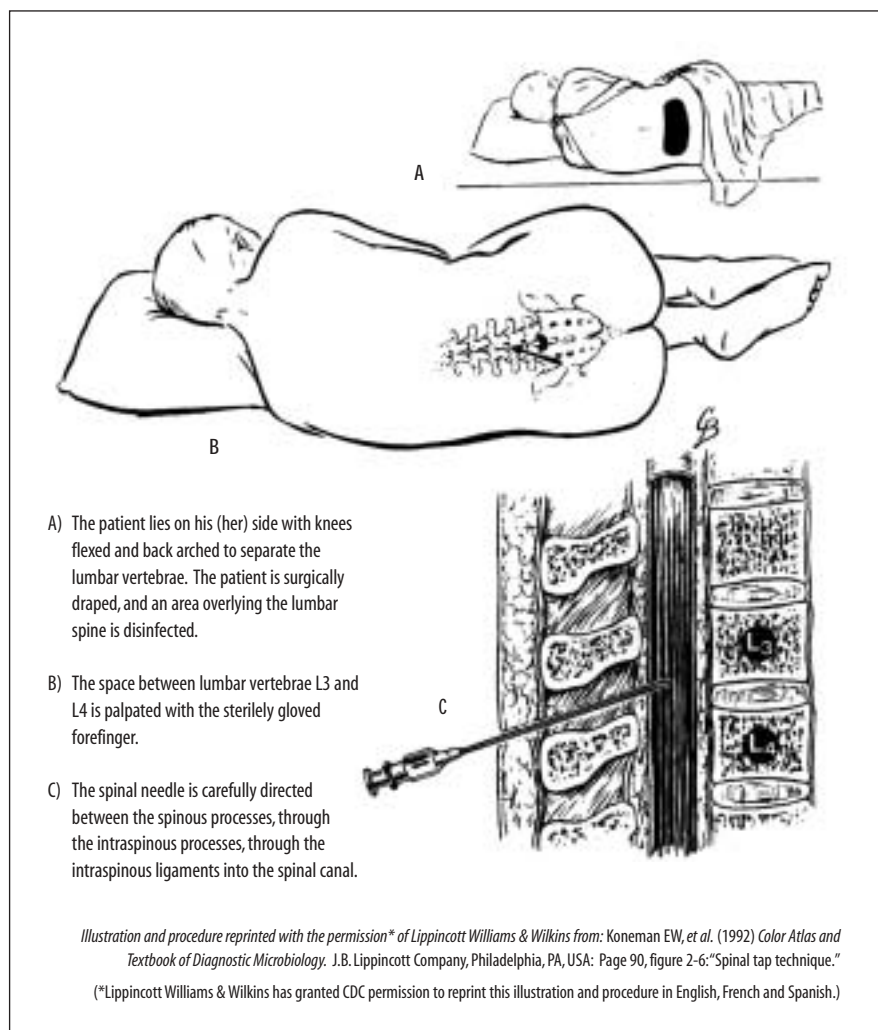
- skin disinfectant
- sterile gauze and adhesive bandages
- lumbar puncture needles: 22 gauge/3.5" for adults; 23 gauge/2.5" for children
- sterile screw-cap tubes
- syringe and needle
- transport container
- Trans-Isolate (T-I) medium (*if CSF cannot be analyzed in the microbiology laboratory immediately*)

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% CO<sub>2</sub> atmosphere (*i.e.*, in a CO<sub>2</sub>-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 IsoVitalX 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% CO<sub>2</sub> 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

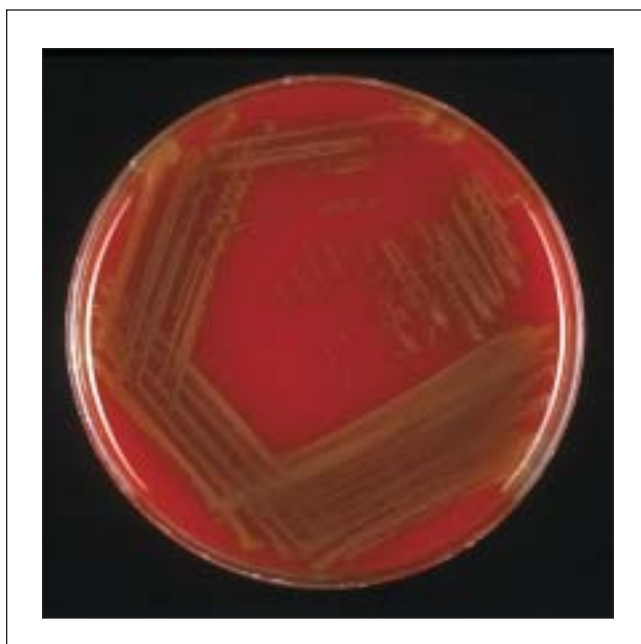


FIGURE 58: Proper streaking and growth of *Haemophilus influenzae* on chocolate agar



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( $\alpha$ )-hemolysis, alpha-prime( $\alpha'$ )-hemolysis and beta( $\beta$ )-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 - l) 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



Growth on sheep blood agar plate (BAP)



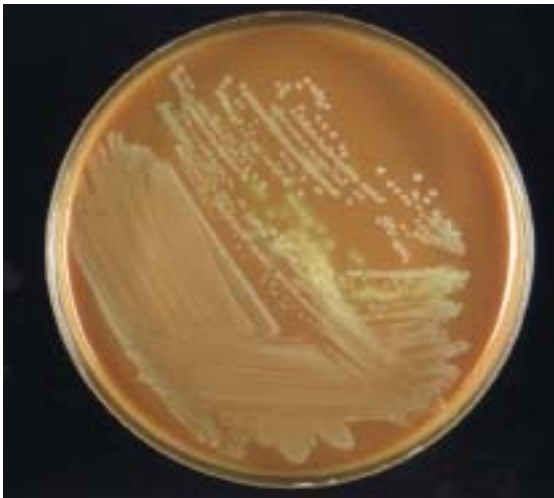
Growth on chocolate agar plate (CAP)

✓ *H. influenzae* was inoculated on the right third of each plate; it grows on CAP but not BAP.

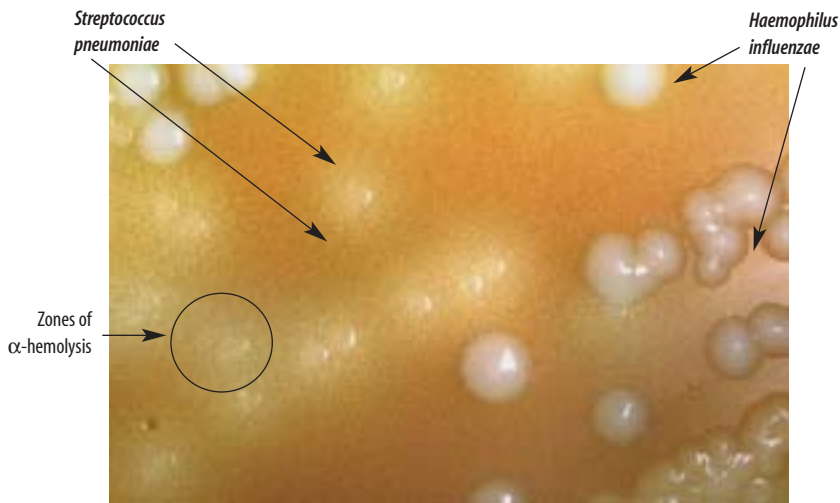
✓ *S. pneumoniae* was inoculated on the top of each plate; it hemolyzes the blood. Note the greenish  $\alpha$ -hemolysis in the blood agar.

✓ *N. meningitidis* colonies (bottom left of each place) appear grayish and have good growth on both media like *S. pneumoniae* does, but no hemolysis occurs.

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  $\alpha$ -hemolysis.

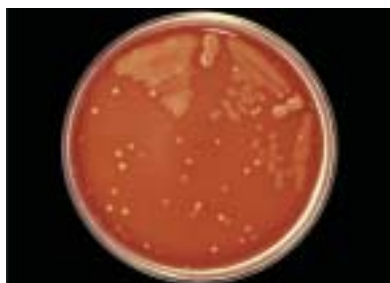
FIGURE 63: Sample worksheet for the presumptive laboratory identification of bacterial agents of pneumonia and meningitis

Specimen number, (type), date	Medium	Appearance of each colony type	Hemolysis ( $\alpha$ , $\beta$ , none)	Gram stain & arrangement	(If Gram + cocci) Optochin ?	(If Gram + cocci) Bile soluble ?	(If G — bacilli & growth on CAP only) X and V Factors?	(If Gram — diplococcus) Oxidase test	(If Gram — diplococcus, Oxidase +) Carbo-hydrate utilization	(H. influenzae, N. meningitidis) Slide serology	Identification
MQP4-30 (blood culture) 22-04-01	Blood agar (BAP)	BAP 1 Small, grayish Flat center	$\alpha$ ha	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 +) type b	H. influenzae
		CAP 2 Small, grayish flat center	$\alpha$ ha	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 ( $\alpha$ ), alpha-prime ( $\alpha'$ ) and beta ( $\beta$ ) hemolysis on sheep blood agar



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



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



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



$\alpha'$ -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  $\alpha'$ -prime ( $\alpha'$ ) 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)



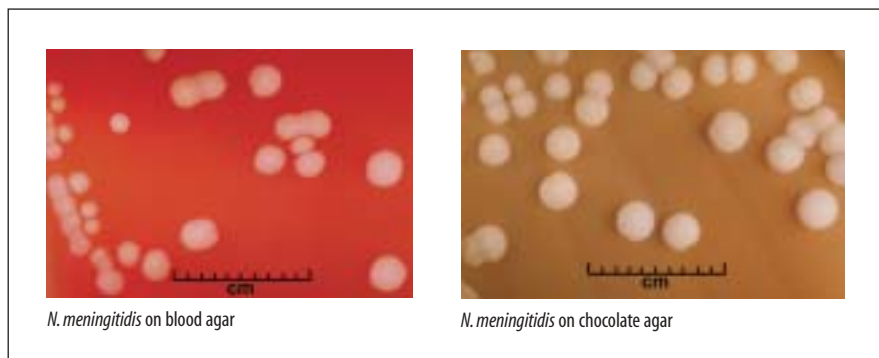
- **Presumptive identification of *S. pneumoniae***

*S. pneumoniae* appears as small, greyish, moist (sometimes mucoid), watery colonies with a greenish zone of  $\alpha$ -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  $\alpha$ -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)

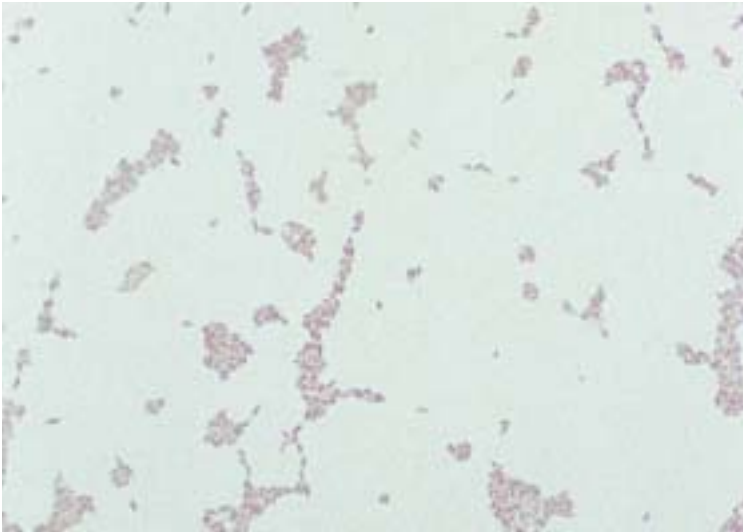


FIGURE 68: *Streptococcus pneumoniae* and *Staphylococcus aureus* growing together on the same blood agar plate



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



Like all other members of the *Enterobacteriaceae*, *S. Typhi* is a gram-negative bacillus (rod).

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)

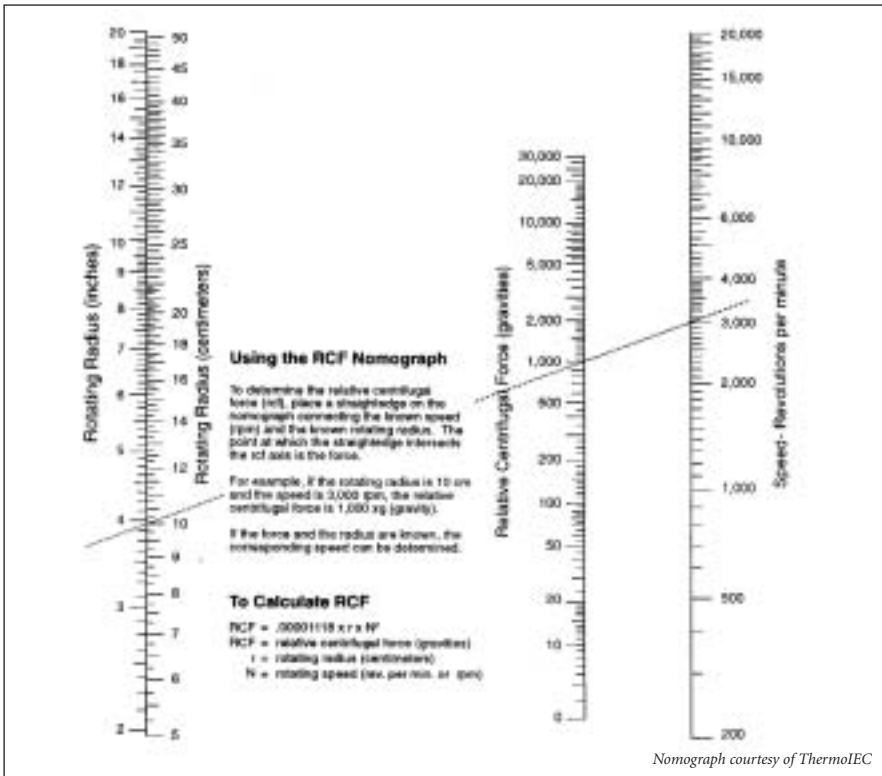
Once the CSF has arrived at the microbiology laboratory, note whether there is more than 1 ml available for analysis. **If less than 1 ml of CSF is available, it should not be centrifuged; instead, the CSF should be plated directly for the Gram stain.**

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).<sup>38</sup> 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

<sup>38</sup> 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)*

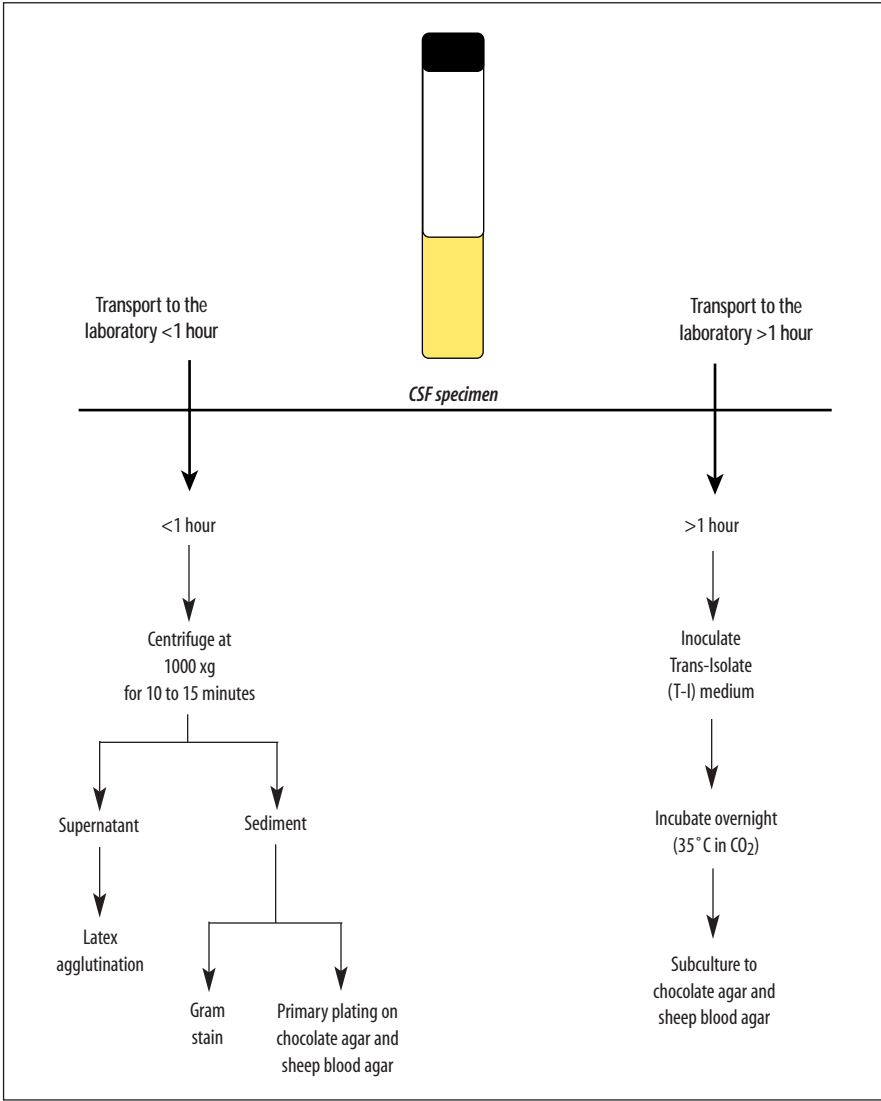
After the CSF has been centrifuged and the sediment well-mixed, a portion of the sediment is Gram stained.

- 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*

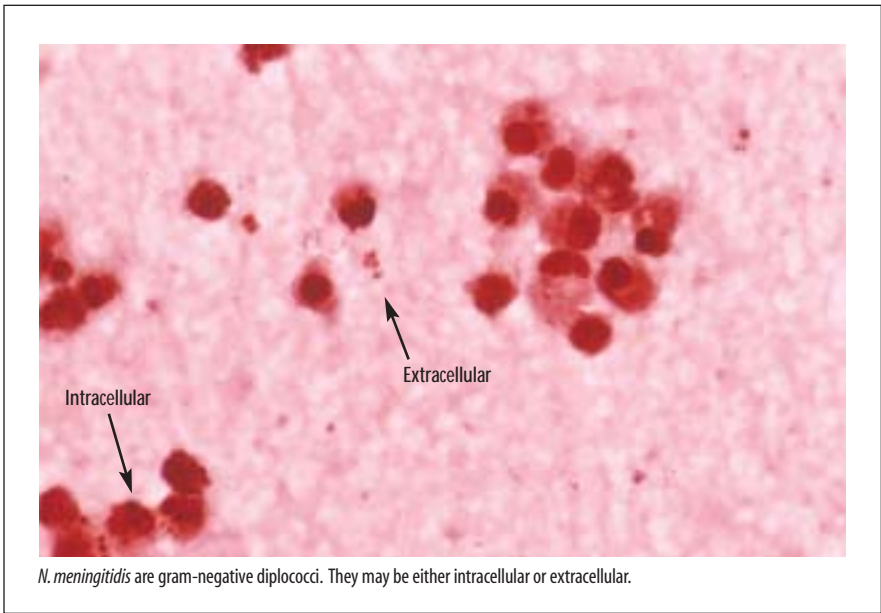


FIGURE 73: Gram stain of cerebrospinal fluid (CSF) with *Streptococcus pneumoniae*

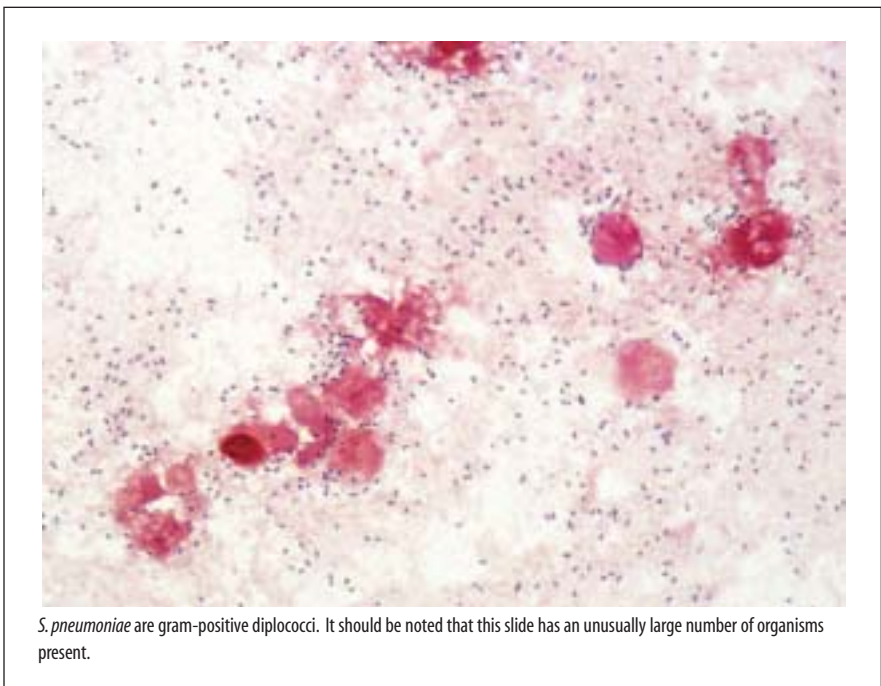
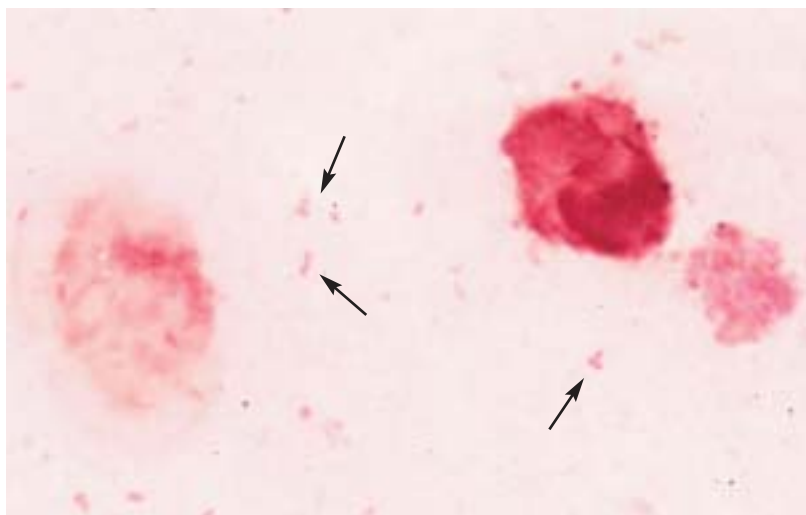


FIGURE 74: Gram stain of cerebrospinal fluid (CSF) with *Haemophilus influenzae*



*H. influenzae* are small gram-negative pleomorphic coccobacilli.

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% CO<sub>2</sub>-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% CO<sub>2</sub>-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 CO<sub>2</sub> 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- $\mu$ l or 10- $\mu$ 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.

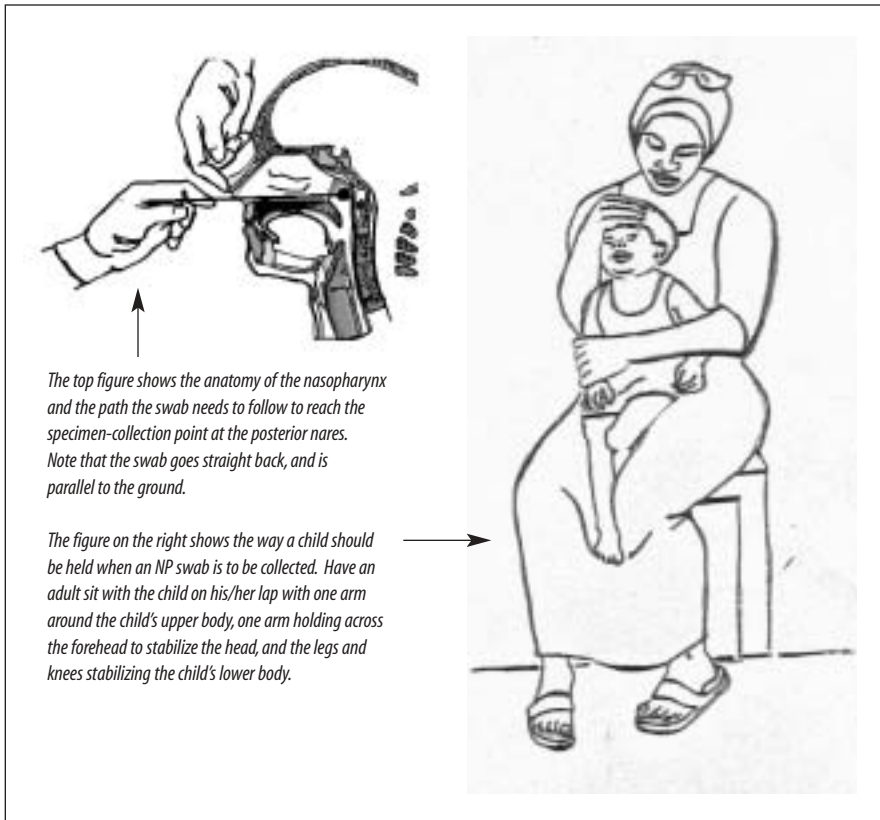
## Skim-milk tryptone glucose glycerol (STGG) transport medium for nasopharyngeal secretions

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  $-70^{\circ}\text{C}$ , if possible.

In some cases, the inoculated STGG medium has been placed on ice for several hours before placing the STGG medium at  $-70^{\circ}\text{C}$  without loss of viable *S. pneumoniae*. Extended storage of inoculated STGG stored at  $-20^{\circ}\text{C}$  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.<sup>39</sup>

<sup>39</sup> 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

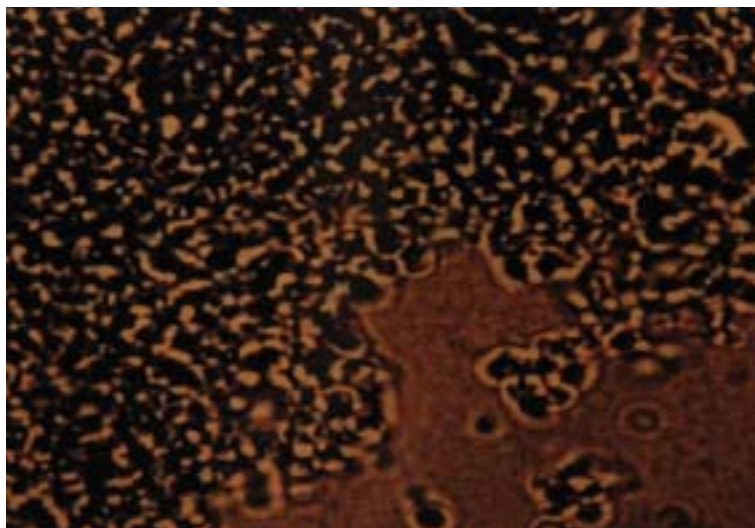
The type and condition of a culture that is received in the laboratory will determine the procedure used to prepare a suitable cell suspension for observation of the Quellung reaction.

- 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 CO<sub>2</sub> 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-mm<sup>2</sup> 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



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

<sup>a</sup> 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.

<sup>b</sup> 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.

<sup>c</sup> Pools G and I do not react with vaccine types and are therefore not included in the checkerboard system.

*Checkerboard table is adapted from Sørensen (1993) and LaLitha et al. (1999)*

## Antimicrobial Susceptibility Testing by Broth Microdilution

**M**inimal 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% CO<sub>2</sub> 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	<b>Note:</b> 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	<b>Note:</b> 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	<b>Note:</b> 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 would be recorded as 0.096 µg/ml, but interpreted as 0.125 µg/ml for the final report.)
0.5 µg/ml	
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 reagent<sup>40</sup> from the tube and place a drop on

<sup>40</sup> 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% CO<sub>2</sub> for 18–22 hours. Use isolated colonies from this plate to set up the antimicrobial susceptibility tests.

Y 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% CO<sub>2</sub> 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 1x10<sup>8</sup> 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% CO<sub>2</sub> 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 CO<sub>2</sub>-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 CO<sub>2</sub>-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 <sub>2</sub> atmosphere (3%–5% CO <sub>2</sub> ) for primary isolation. Some strains have an obligate requirement for CO <sub>2</sub> , whereas other strains lose this requirement on subculture. Use a CO <sub>2</sub> -incubator or a candle-extinction (candle) jar. Re-light the candle each time the candle-jar is opened to add plates. (Note: 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 (IsoVitalX 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	<p><i>N. gonorrhoeae</i> is sensitive to some materials found in swabs.</p> <p>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:</p> <ol style="list-style-type: none"> <li>1) synthetic swabs often do not absorb liquid easily; and,</li> <li>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.</li> </ol>

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 CO<sub>2</sub>-enriched atmosphere is more important than incubating them at 35°–36.5°C. Inoculated media may be held at room temperature in a CO<sub>2</sub>-enriched atmosphere in candle-extinction jars or an alternative CO<sub>2</sub>-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 CO<sub>2</sub>-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 CO<sub>2</sub>-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, CO<sub>2</sub>-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 CO<sub>2</sub>-enriched atmosphere for primary isolation.**

Although some strains lose their requirement for a CO<sub>2</sub>-enriched atmosphere for growth in subculture, some strains have an obligatory requirement for CO<sub>2</sub> which is not lost on subculture. CO<sub>2</sub> incubators should be used if large numbers of specimens must be processed. If a CO<sub>2</sub> incubator is not available, culture plates may be incubated either with commercial CO<sub>2</sub>-generating systems (producing a concentration of 3%–5% CO<sub>2</sub>) 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% CO<sub>2</sub> 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 CO<sub>2</sub> 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"> <li>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.)</li> <li>2. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO<sub>2</sub>-enriched atmosphere or place in a candle-extinction jar for transport to the laboratory.</li> <li>3. Prepare a smear for Gram stain.</li> </ol>	<ol style="list-style-type: none"> <li>a. Specimen should be collected no sooner than 1 hour after the patient has urinated.</li> <li>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.</li> <li>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>.</li> </ol>
Cervix	<ol style="list-style-type: none"> <li>1. Insert a non-lubricated speculum into the vagina so the cervix can be seen.</li> <li>2. Use a swab to remove mucus and secretions from the cervical os; discard this swab.</li> <li>3. Use a sterile swab to gently but firmly sample the endocervical canal.</li> <li>4. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO<sub>2</sub> environment or place in a candle-extinction jar for transport to the laboratory.</li> </ol>	<ol style="list-style-type: none"> <li>a. Ensure that the swab used to collect the endocervical specimen does not touch the vaginal walls during the procedure.</li> <li>b. In pre-pubescent girls, vaginal specimens may be substituted for endocervical specimens.</li> </ol>
Vagina (only in prepubescent females)	<ol style="list-style-type: none"> <li>1. Wipe any excessive secretions or discharge.</li> <li>2. Rub a Dacron or rayon swab against the mucus membranes of the posterior vaginal wall for 10–15 seconds to absorb secretions.</li> <li>3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO<sub>2</sub> environment or place in a candle-extinction jar for transport to the laboratory.</li> </ol>	<ol style="list-style-type: none"> <li>a. Collect the specimen from the vaginal orifice if the hymen is intact.</li> </ol>

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"> <li>1. Insert a sterile swab approximately 1 inch beyond the anal sphincter.</li> <li>2. Gently rotate the swab to sample the anal crypts.</li> <li>3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO<sub>2</sub> environment or place in a candle-extinction jar for transport to the laboratory.</li> </ol>	<ol style="list-style-type: none"> <li>a. Discard anorectal swabs that are contaminated with fecal material; obtain a second specimen.</li> </ol>
Pharynx (if a history of oral-genital or oro-anal exposure exists)	<ol style="list-style-type: none"> <li>1. Depress tongue with a tongue depressor.</li> <li>2. Use a sterile swab to sample the posterior pharynx, tonsils, and inflamed areas.</li> <li>3. Immediately inoculate MTM, ML, or equivalent selective medium for <i>N. gonorrhoeae</i>. Incubate inoculated plates immediately in a CO<sub>2</sub> environment or place in a candle-extinction jar for transport to the laboratory.</li> </ol>	—
Conjunctiva	<ol style="list-style-type: none"> <li>1. Moisten two swabs with sterile saline.</li> <li>2. Swab each eye with a separate swab by rolling the swab over the conjunctiva.</li> <li>3. Immediately inoculate each swab onto a non-selective plate (e.g., chocolate agar) and incubate inoculated plates immediately in a CO<sub>2</sub> environment or place in a candle-extinction jar for transport to the lab.</li> <li>4. Smear each swab onto a separate slide for Gram stain.</li> </ol>	<ol style="list-style-type: none"> <li>a. Sample both conjunctivae if possible, but if prohibitively expensive, culture infected conjunctiva.</li> <li>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.</li> <li>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>.</li> </ol>

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 CO<sub>2</sub>-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 CO<sub>2</sub>-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 CO<sub>2</sub>-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 CO<sub>2</sub>-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.

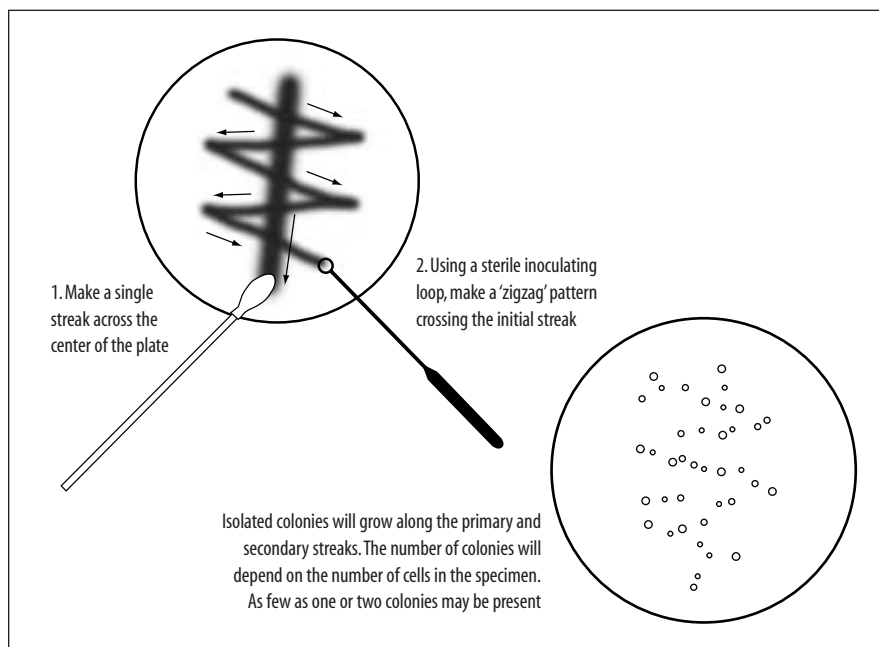
It should be noted that, when Gram staining, clumps of cells might appear a dark color due to the retention of crystal violet in the clump, even after proper decolorization, leading to the misinterpretation of some gram-negative cells as gram-positive. However, attempts to adequately decolorize the clumps 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"> <li>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"> <li>0.5-mm colonies tend to be high-convex in elevation</li> <li>1.0-mm colonies tend to be low-convex in elevation</li> </ul> </li> <li>Fastidious strains of <i>N. gonorrhoeae</i> produce atypically small, “pinpoint” colonies (~0.25 mm in diameter) compared with other, less fastidious gonococcal strains.</li> </ul>
<i>N. meningitidis</i>	<ul style="list-style-type: none"> <li>Colonies are of similar appearance on gonococcal selective and nonselective media: pinkish brown and translucent, with smooth consistency and defined margins</li> <li>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>).</li> <li>Colonies of encapsulated serogroups A and C strains may be mucoid.</li> </ul>
<i>N. lactamica</i> <i>N. cinerea</i> <i>N. polysaccharea</i> <i>K. denitrificans</i>	<ul style="list-style-type: none"> <li>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"> <li>Colonies of <i>N. lactamica</i> may have a yellowish pigment.</li> <li>Colonies of <i>N. cinerea</i> may have a brownish pigment.</li> </ul> </li> </ul>
<i>N. subflava</i> biovars <i>N. sicca</i> <i>N. mucosa</i>	<ul style="list-style-type: none"> <li>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"> <li>Colonies of <i>N. subflava</i> bv. <i>perflava</i> and <i>N. mucosa</i> are convex and glistening.</li> <li>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.</li> <li>Colonies of <i>N. sicca</i> may adhere to the agar surface and become wrinkled with prolonged incubation.</li> </ul> </li> </ul>
<i>M. catarrhalis</i>	<ul style="list-style-type: none"> <li>Colonies are usually 1.0–3.0 mm in diameter, opaque, friable (dry) in consistency, and pinkish-brown. <ul style="list-style-type: none"> <li>Colonies may be moved intact over the surface of the medium with an inoculating loop.</li> <li>Colonies disintegrate in chunks when broken with a loop.</li> </ul> </li> </ul>

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*

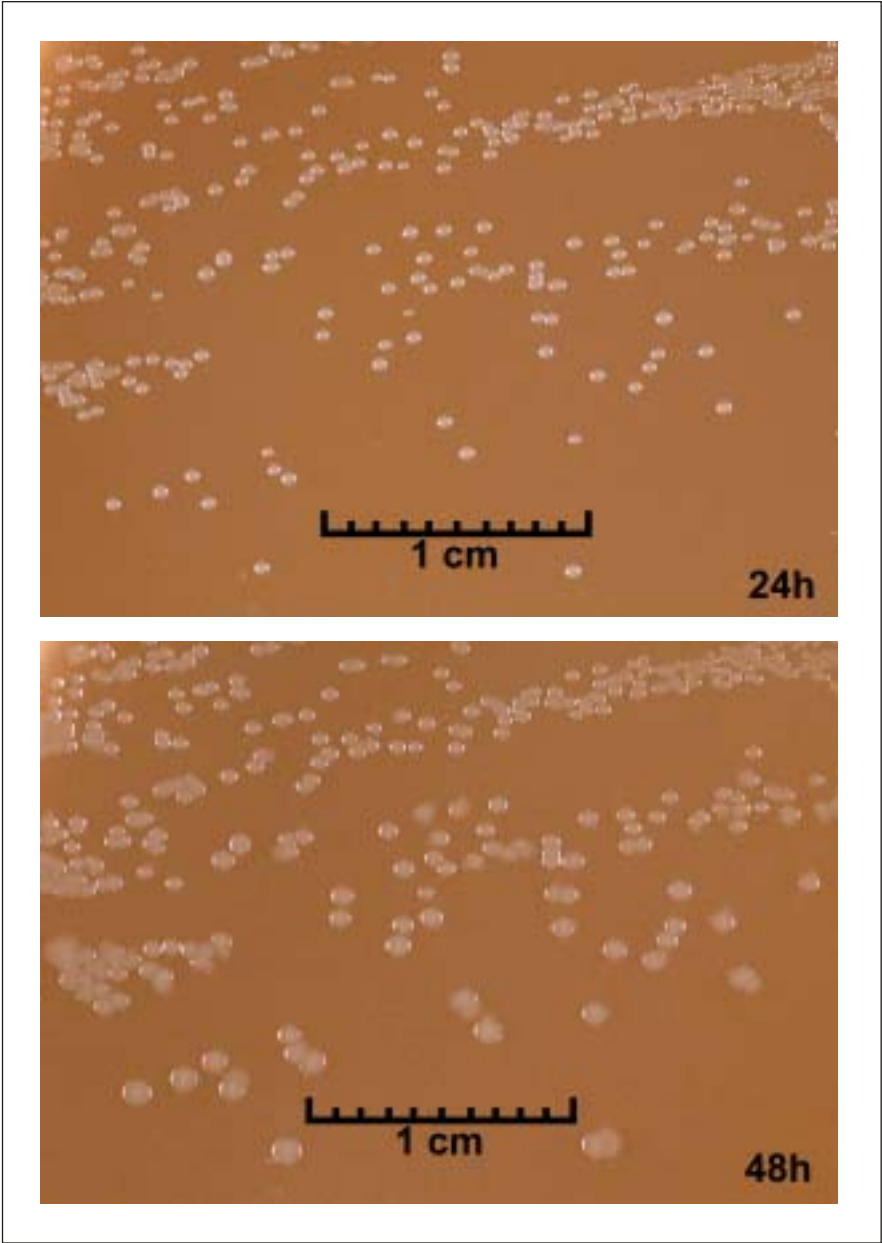
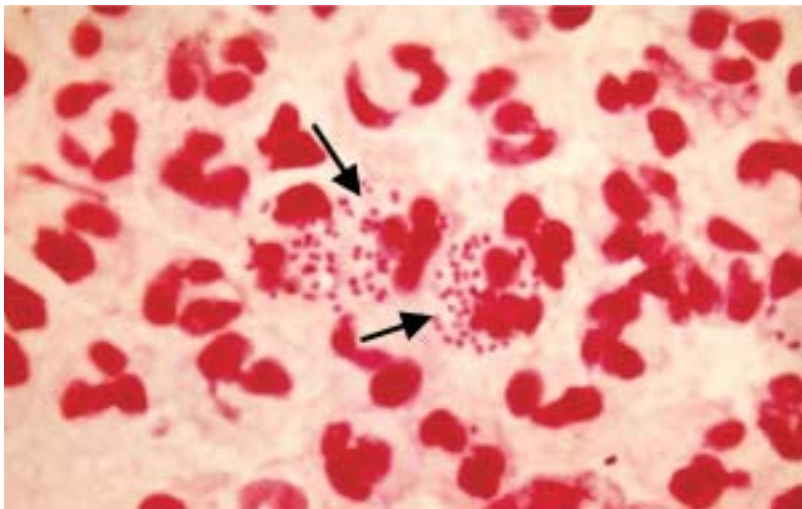
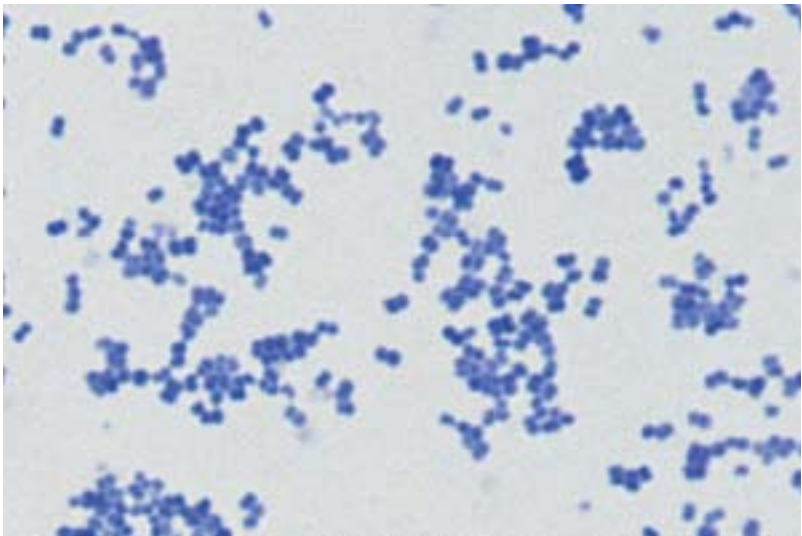


FIGURE 80: Gram stain and simple single Loeffler's methylene blue stain of *Neisseria gonorrhoeae*



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  $\alpha$ -hemolysis.

Recognition of pure colonies of *N. gonorrhoeae* is often easier after incubation at 35°–36.5°C in a CO<sub>2</sub>-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 CO<sub>2</sub>-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  $-70^{\circ}\text{C}$ . 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).

[illegible]

## 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> )
<b>Antimicrobial susceptibility test supplies for 100 <i>Shigella</i> isolates (for national reference laboratory only)</b>			
• Mueller-Hinton agar	2	2	2 x 500 grams
• Disposable Petri dishes	2	2	200 dishes
• Trimethoprim-sulfamethoxazole [1.25 / 23.75 -µg disks]	2	2	200 disks
• Chloramphenicol [30-µg disks]	2	2	200 disks
• Ampicillin [10-µg disks]			200 disks
• Nalidixic acid [30-µg disks]	2	2	200 disks
• Ciprofloxacin [5-µg disks]	2	2	100 disks
• NCCLS control strain <i>E. coli</i> ATCC 25922	2	2	(Required)
• 0.5 McFarland turbidity standard	2	2	(Required)
• Sterile cotton swabs	2	2	200 swabs
• Sterile saline	2	2	(Required)
• Forceps	2	2	(Required)
• 95% alcohol for flaming	2	2	(Required)
• Calipers (or ruler on a stick)	2	2	(Required)
• Inhibition zone diameter criteria chart <i>[for interpretation per NCCLS]</i>	2	2	(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	2	(Required)	(Required)
pH paper or pH meter	2	(Required)	(Required)
Petri plates (9-cm)	2	500 plates	5 x 500 plates
Test tubes (e.g., 13 x 100-mm or 16 x 125-mm)	2	1 000 tubes	5 x 1 000 tubes
Materials and postage (for the production & dissemination of reports)	2	(Required)	(Required)
<b>Antimicrobial susceptibility test supplies for 100 <i>V. cholerae</i> isolates (for national reference laboratory only)</b>			
• Mueller-Hinton agar	2	2	2 x 500 grams
• Disposable Petri plates	2	2	200 plates
• Trimethoprim-sulfamethoxazole [1.25 / 23.75 -µg disks]	2	2	200 disks
• Tetracycline [30-µg disks]	2	2	200 disks
• Nalidixic acid [30-µg disks]	2	2	200 disks
• Ciprofloxacin [5-µg disks]	2	2	100 disks
• NCCLS control strain <i>E. coli</i> ATCC 25922	2	2	(Required)
• 0.5 McFarland turbidity standard	2	2	(Required)
• Sterile cotton swabs	2	2	200 swabs
• Sterile saline	2	2	(Required)
• Forceps	2	2	(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> )
• 95% alcohol for flaming	2	2	(Required)
• Calipers (or ruler on a stick)	2	2	(Required)
• Inhibition zone diameter criteria chart [for interpretation per NCCLS]	2	2	(Required)

## 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.

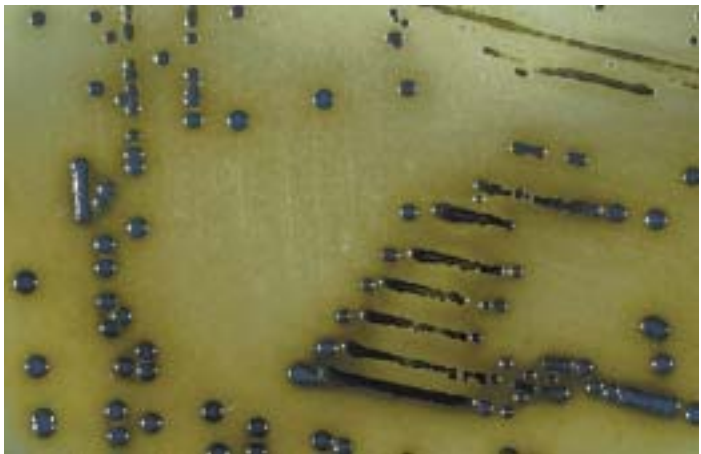
BS plates must be freshly prepared (Appendix 2) and used within 36 hours for isolation of *S. Typhi*. A rectal swab or stool swab may be used to inoculate BS agar by seeding an area approximately 1 inch in diameter on the agar, after which the plate is streaked for isolation. After seeding the plate,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

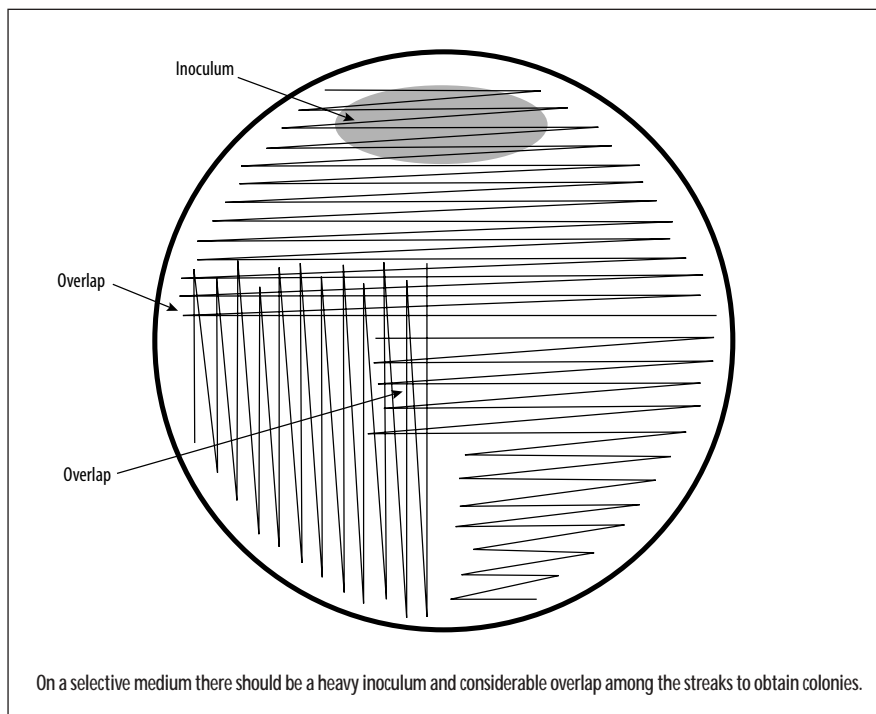
Fecal specimens should be plated as soon as possible after arrival in the laboratory. Selective media may be inoculated with a single drop of liquid stool or fecal suspension. Alternatively, a rectal swab or a fecal swab may be used. If a swab is used to inoculate selective media, an area approximately 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 <sup>a,b</sup>	Figure 88
Xylose lysine desoxycholate (XLD)	Red or colorless	1 – 2 mm <sup>a,c</sup>	Figures 85, 86, and 87
Desoxycholate citrate agar (DCA)	Colorless	2 – 3 mm <sup>a</sup>	~
Hektoen enteric agar (HE)	Green	2 – 3 mm <sup>a</sup>	~

<sup>a</sup> *S. dysenteriae* 1 colonies may be smaller.

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

<sup>c</sup> *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

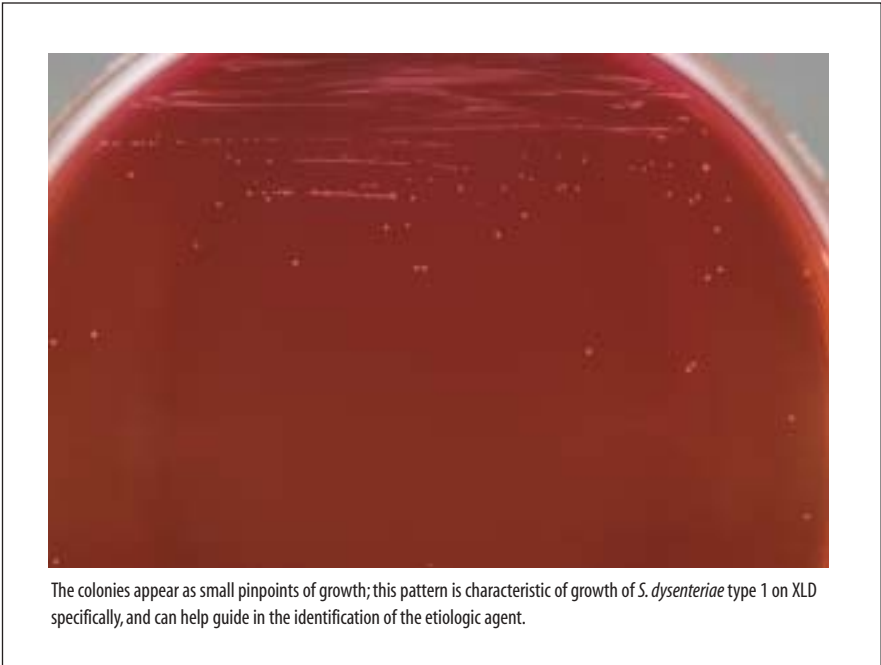
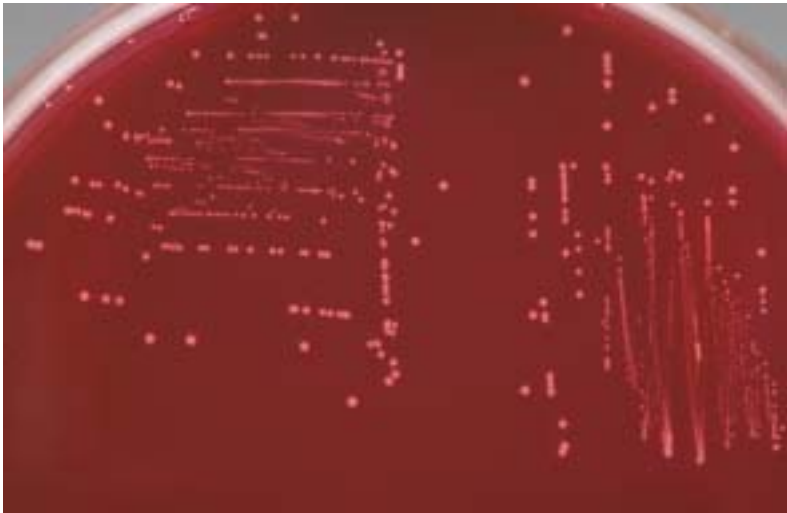


FIGURE 86: *Shigella flexneri* colonies on xylose lysine desoxycholate (XLD) agar



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. parahaemolyticus*) 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.<sup>41</sup> 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

<sup>41</sup> 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.

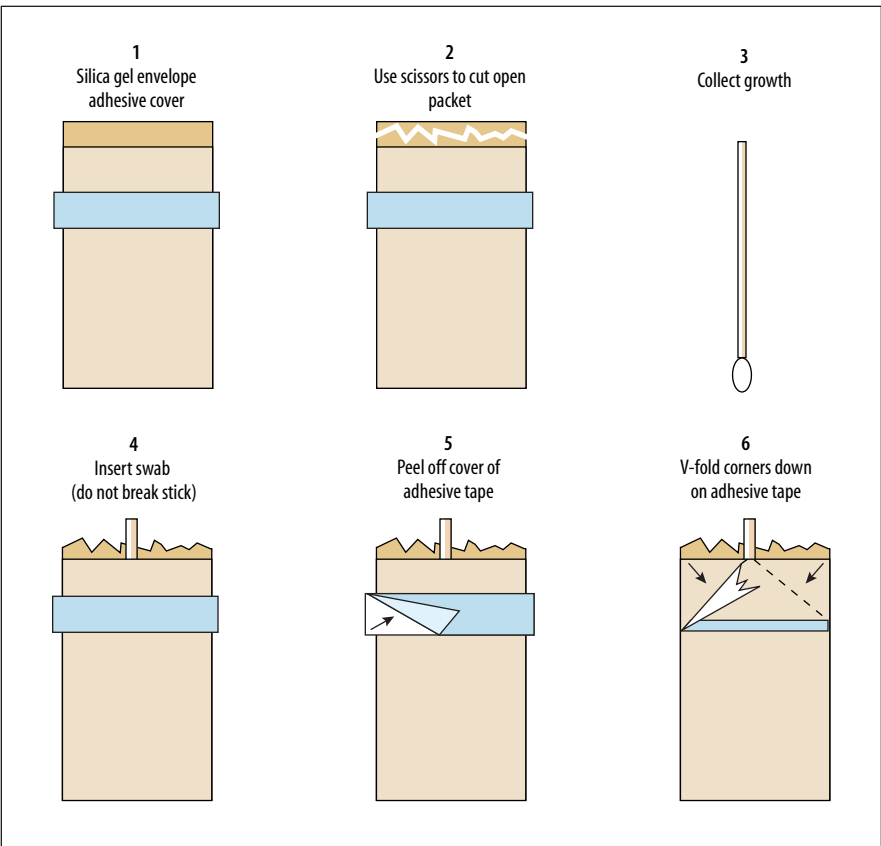
### Long-term storage of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*

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 CO<sub>2</sub> 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 CO<sub>2</sub>-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 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.) 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  $-70^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$ ; 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 CO<sub>2</sub>) 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](mailto: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

<i>Package Type:</i>								(Figure within table)
Diagnostic Specimens	3		3					Figure A
Diagnostic Specimens on Dry Ice	3		3	3				Figure B
Infectious Substance ...with less than 50 ml ...with 50 ml or more	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	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


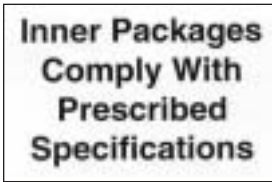
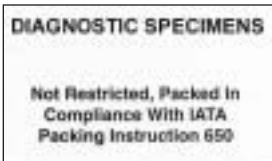
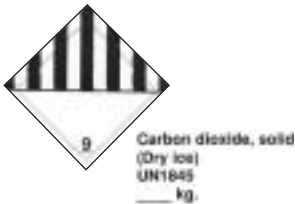

	<p>This orientation label should clearly mark which side is 'Up.' Two labels are required on all boxes, each one on opposite sides of the package.</p>
	<p>This marking must appear on an overpack when the regulations require the use of packagings bearing UN Specification Markings.</p>
	<p>This marking is required when shipping diagnostic specimens.</p>
	<p>These two labels are required when shipping a substance or specimen on dry ice.</p>
	<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*



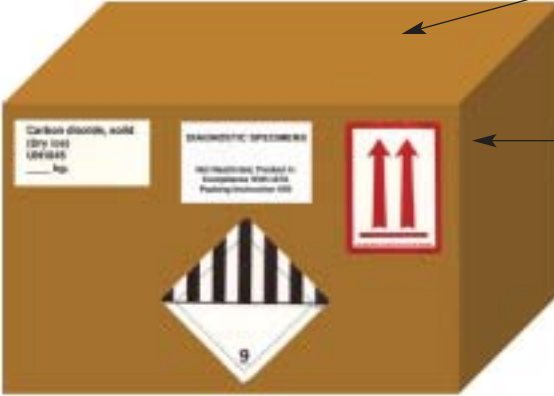
	<p>This label is required when shipping <math>\geq 50</math> ml of an infectious substance.</p>
<p><i>Figure A: Package with diagnostic specimens</i></p>	
	<p>Surface to which air waybill and/or address labels are affixed</p> <p>Must have two "up" arrows on opposite sides</p>
<p><i>Figure B: Package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

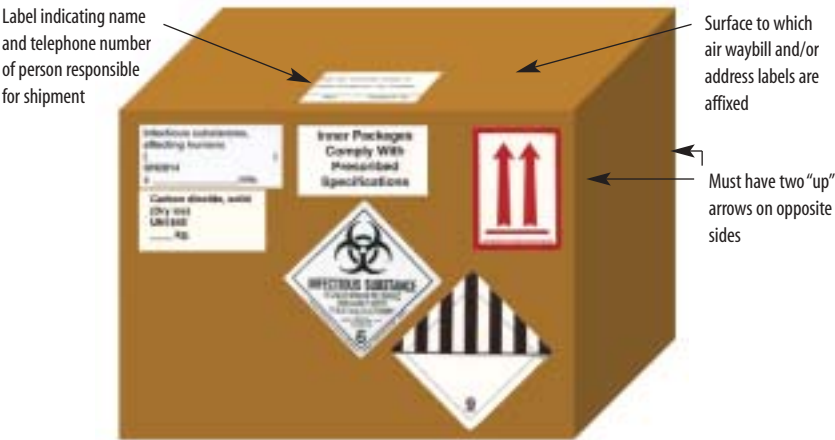


Figure D: Overpack with ≥ 50 ml of infectious substance



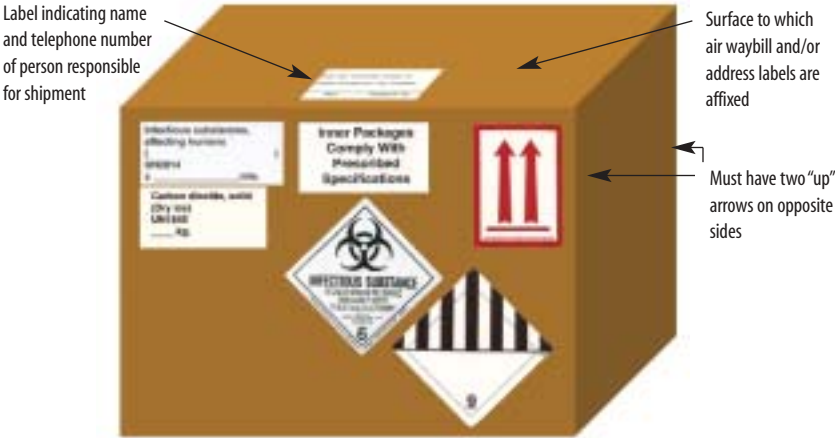
TABLE 36b: *continued*

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

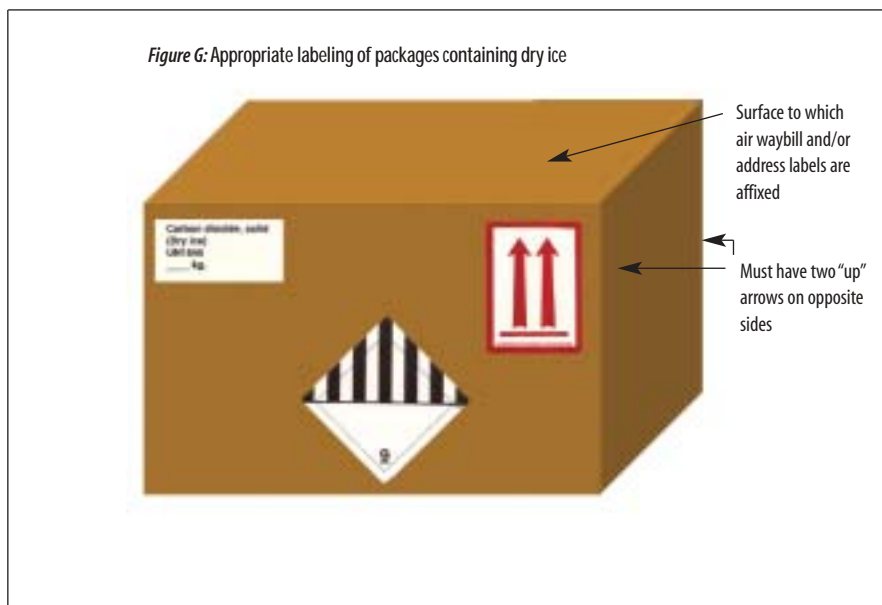


You must use UN certified 6.2 Infectious Substances Packaging.

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



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  $\geq 50$  ml.

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

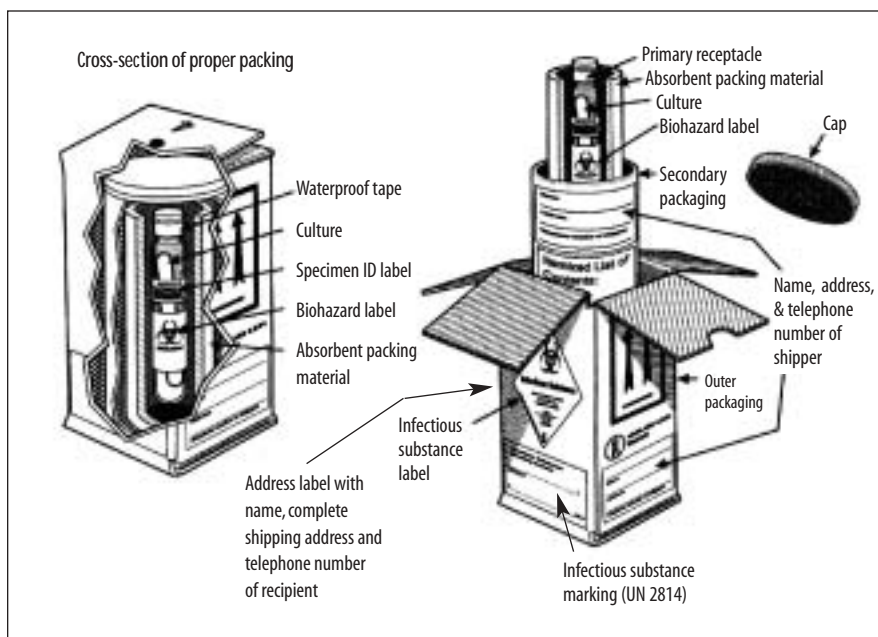
The packaging requirements for transport of infectious substances are illustrated in Figure 91.

### ***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



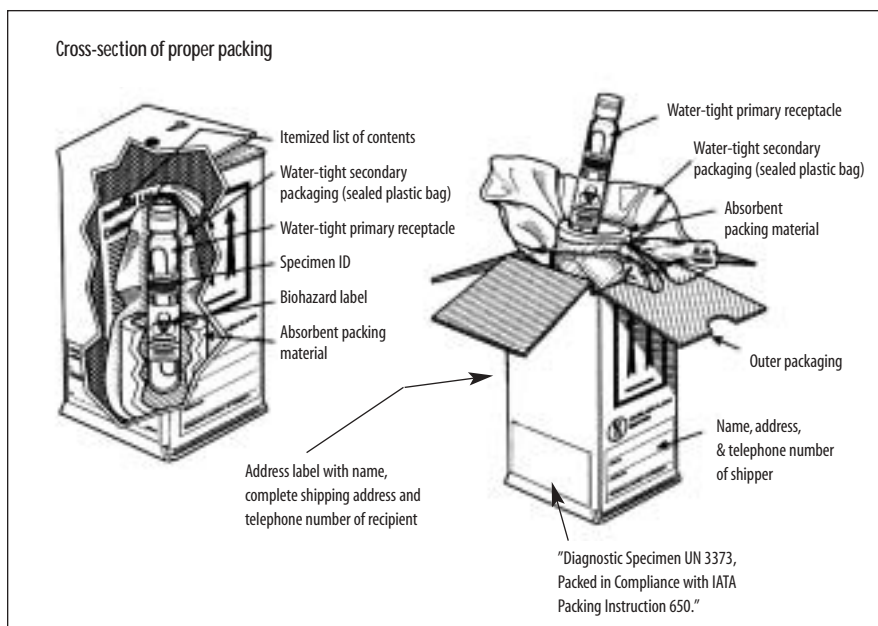
- Be marked with the diagnostic specimens statement on the outside of the outer container: “Diagnostic specimen. UN 3373. Packed in compliance with IATA Packing Instruction 650.” Note that this marking can be written by hand and does not require a special adhesive label.
- 3 If being shipped by air, 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 (CO<sub>2</sub>)***

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 [CO<sub>2</sub>] 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 (Provide at least two copies to the airline)

Shipper: Name of Shipper, Company Name, Complete address (no P.O. Boxes), Telephone number (include area code), Person responsible (name and telephone)

Air Waybill No. (use Air Waybill number of package), Page 1 of Pages (REQUIRED), Shipper's Reference Number (optional)

Consignee: Name of Recipient, Company Name, Complete address (Not a P.O. Box), Telephone number (include area code), Person responsible (name and telephone)

Cross-out choice that does NOT apply (infectious substances are usually non-radioactive), red markings are REQUIRED

Two completed and signed copies of this Declaration must be handed to the operator

TRANSPORT DETAILS: This shipment is within the limits prescribed for limited quantities, Airport of Origin: City, State, Country, AIRCRAFT AND CARGO AIRCRAFT ONLY, Airport of Destination: City, State, Country

WARNING: 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.

NATURE AND QUANTITY OF DANGEROUS GOODS

Dangerous Goods Identification				Quantity and Type of packing	Packing Inst.	Authorization
Proper Shipping Name	Class or Division	ID or UN No.	Packing Group			
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	
				OVERPACK USED		

Additional Handling Information: Prior arrangements as required by the ICAO and IATA Dangerous Goods Regulations 1.3.3.1 have been made (REQUIRED)

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/placarded, and are in all respects in proper condition for transport according to applicable international and national governmental regulations.

Signature/Title of Shipper: Shipper's name, Title, company name, Place and Date: City, State, Country, Date shipped, Signature: Shipper's signature



## 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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### **BD (Becton, Dickinson and Co.)**

*also includes products from:*

- **BBL** (*internet catalogue*)  
<http://catalog.bd.com/scripts/catalog.exe>
- **Difco** (*internet catalogue*)  
<http://www.bd.com/industrial/difco/manual.asp>

### **BD Microbiology Systems**

7 Loveton Circle  
Sparks, Maryland 21152 USA

*Phone:* (+1) 410 316 4000

*Fax:* (+1) 410 316 4723

### **BD Worldwide**

House of Vanguard  
Chiromo Road, Westlands  
4th Floor, Wing B, P.O. Box 76813  
Nairobi, Kenya

*Phone:* (+254) 2 44 96 09

*Fax:* (+254) 2 44 96 19

### **BD Diagnostics Systems, Asia Limited**

5th Floor, Signature Tower South City  
Gurgaon – 122016  
Haryana, India

*Phone:* (+91) 124 638 3566

*Fax:* (+91) 124 638 3224

*E-mail:* [bd\\_india@bd.com](mailto:bd_india@bd.com)

### **BD Chile**

Carretera General San Martin 16500  
Sitio 33, Colina (Casilla 16273 – Correo 9)  
Santiago, Chile

*Phone:* (+56) 2 460 0380

*Fax:* (+56) 2 460 0306

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*Fax:* (+1) 314 731 8700

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*Fax:* (+33) 4 78 87 20 90  
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*Internet:* <http://www.dht-online.co.uk/>

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Fisher Scientific, Puerto Rico  
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Barrio Montellano  
Cayey, Puerto Rico 00737 USA  
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*Fax:* (+1) 787 738 4600

Fisher Scientific International, Inc.  
3970 Johns Creek Court  
Suite 500  
Suwanee, GA 30024 USA  
*Phone:* (+1) 770 871 4500  
*Fax:* (+1) 770 871 4600

Europe/Middle East/Africa Headquarters  
Fisher Scientific Overseas Marketing, Inc.  
46 Queen Anne Street  
London W1M 9LA, United Kingdom  
*Phone:* (+44) 171 935 4440  
*Fax:* (+44) 171 935 5758

*Listing of additional locations:*  
<http://www.fishersci.com.sg/contact.html>

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**Merck & Co KGaA**

*Electronic listing of  
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Frankfurter Strasse 250

64293 Darmstadt, Germany

*Phone:* (+49) 6151 720

*Fax:* (+49) 6151 722000

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1 Friesland Drive

Longmeadow Business Estate

Modderfontein, Gauteng, South Africa

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**Pastorex**

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**Wellcome Diagnostics**

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Glaxo Wellcome UK Ltd.,  
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## 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](mailto: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](mailto: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.abbiodisk.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](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](mailto: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>)

*Manual of Clinical Microbiology*. American Society for Microbiology - Washington, DC USA: 1992

*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

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