

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, CO₂-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)¹⁸ 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.

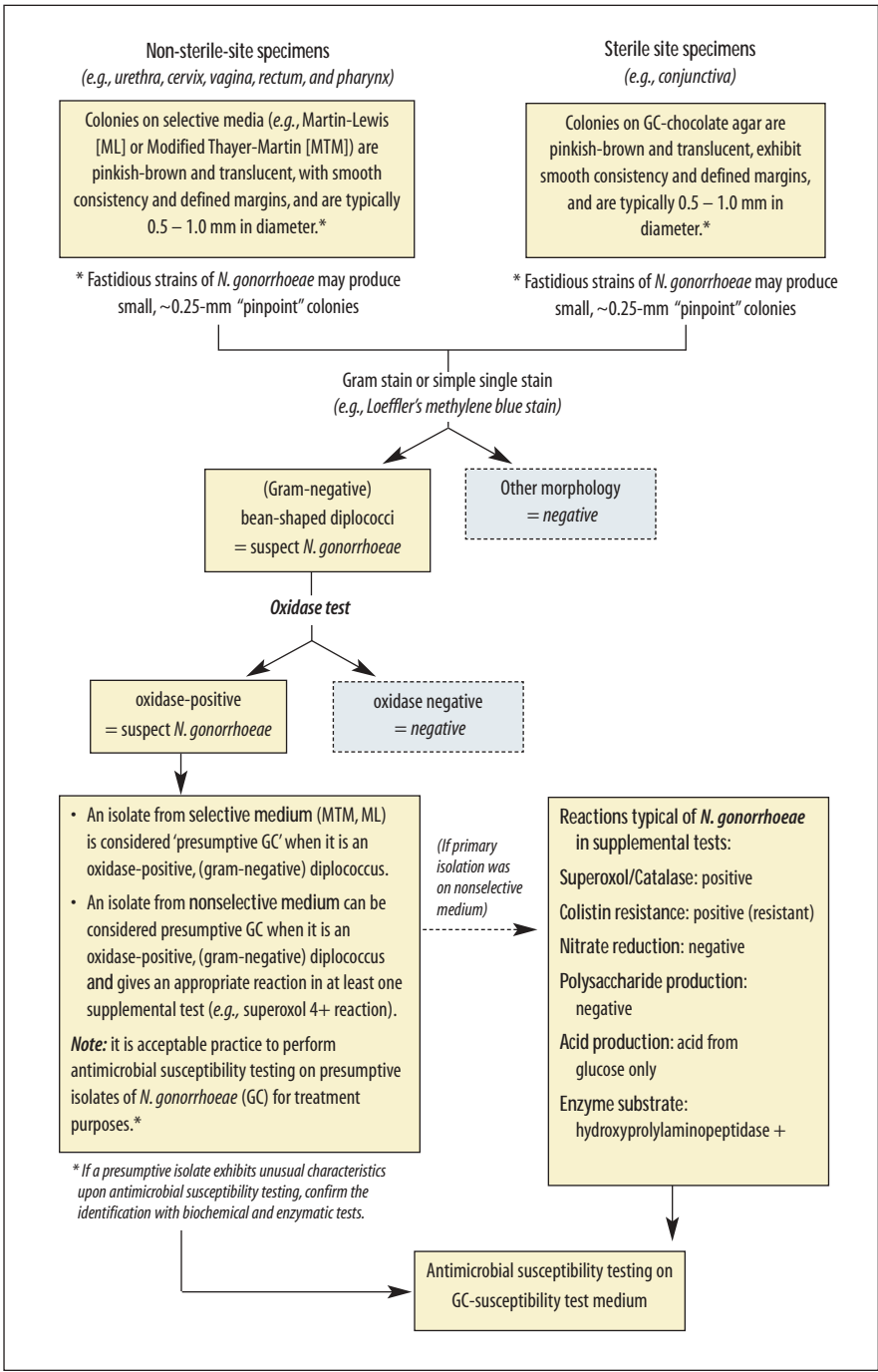
Perform an oxidase test on growth of representative colonies that stained as (gram-negative) diplococci. Because the oxidase reagent is toxic for bacteria, it is recommended to perform the oxidase test on a sterile swab and not directly on the culture plate, particularly if there are only a few suspect 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.¹⁸ (See Figure 20).

¹⁸ 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*



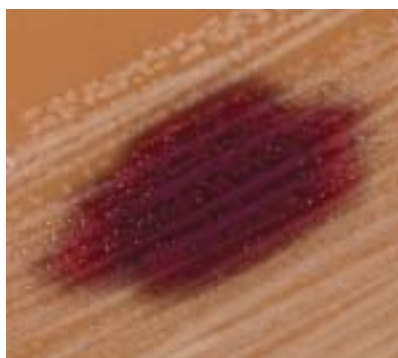
- **Moistened filter paper method for Kovac's oxidase test**

- 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.¹⁸ (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.¹⁹

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

¹⁹ 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*

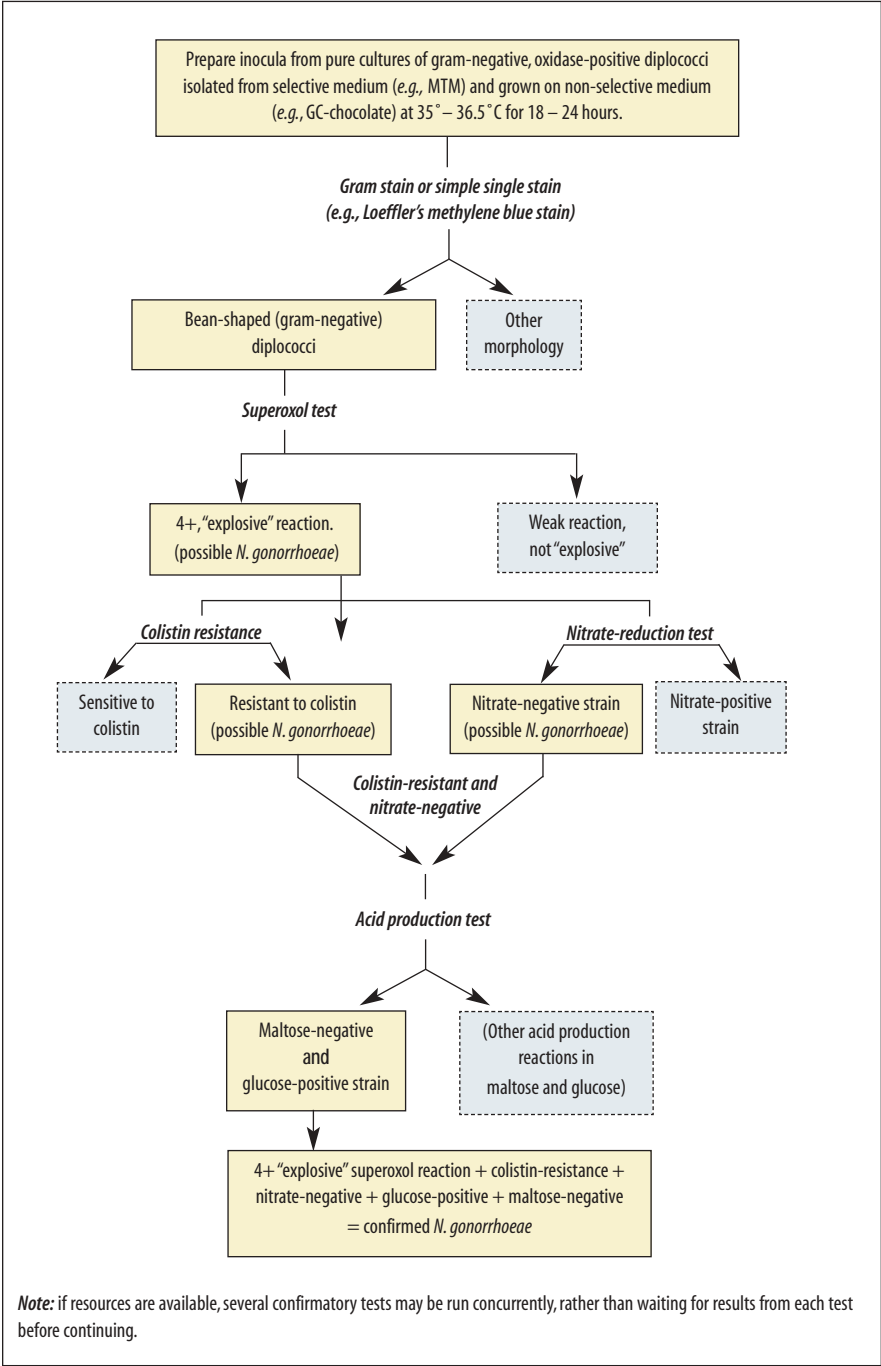


TABLE 6: Results of biochemical and enzymatic tests for *Neisseria gonorrhoeae* and related species with similar colonial morphology

Species	Cell morphology	Superoxol { <i>Catalase</i> }	Colistin	Production of acid from:				Reduction of NO ₃ (Nitrate)	Polysaccharide from sucrose
				GLU	MAL	LAC	SUC		
Test isolate:									
<i>N. gonorrhoeae</i> ^a	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> ^b	GND	2+	{+}	(R)	-	-	-	-	-
<i>N. polysacharea</i>	GND	1+ to 3+	{+}	(R)	+	-	-	-	-
<i>N. subflava</i> ^b	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> ^c	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).

^a 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).

^b 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.

^c 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">• <i>N. gonorrhoeae</i> (ATCC 49226) produces acid from glucose• <i>N. meningitidis</i> (ATCC 13077) produces acid from glucose and maltose• <i>N. lactamica</i> (ATCC 23970) produces acid from glucose, maltose, and lactose• <i>N. mucosa</i> (ATCC 19696) produces acid from glucose, maltose, and sucrose• <i>N. cinerea</i> (ATCC 14685) glucose negative, but may produce a weak glucose reaction; does not produce acid from the other sugars.	
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">• <i>N. gonorrhoeae</i> (ATCC 49226) produces hydroxyprolylaminopeptidase.• <i>N. meningitidis</i> (ATCC 13077) produces γ-glutamylaminopeptidase.• <i>N. lactamica</i> (ATCC 23970) produces β-galactosidase.• <i>M. catarrhalis</i> (ATCC 25238) produces none of these enzymes.	

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 (H_2O_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% H_2O_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.²⁰
- 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.

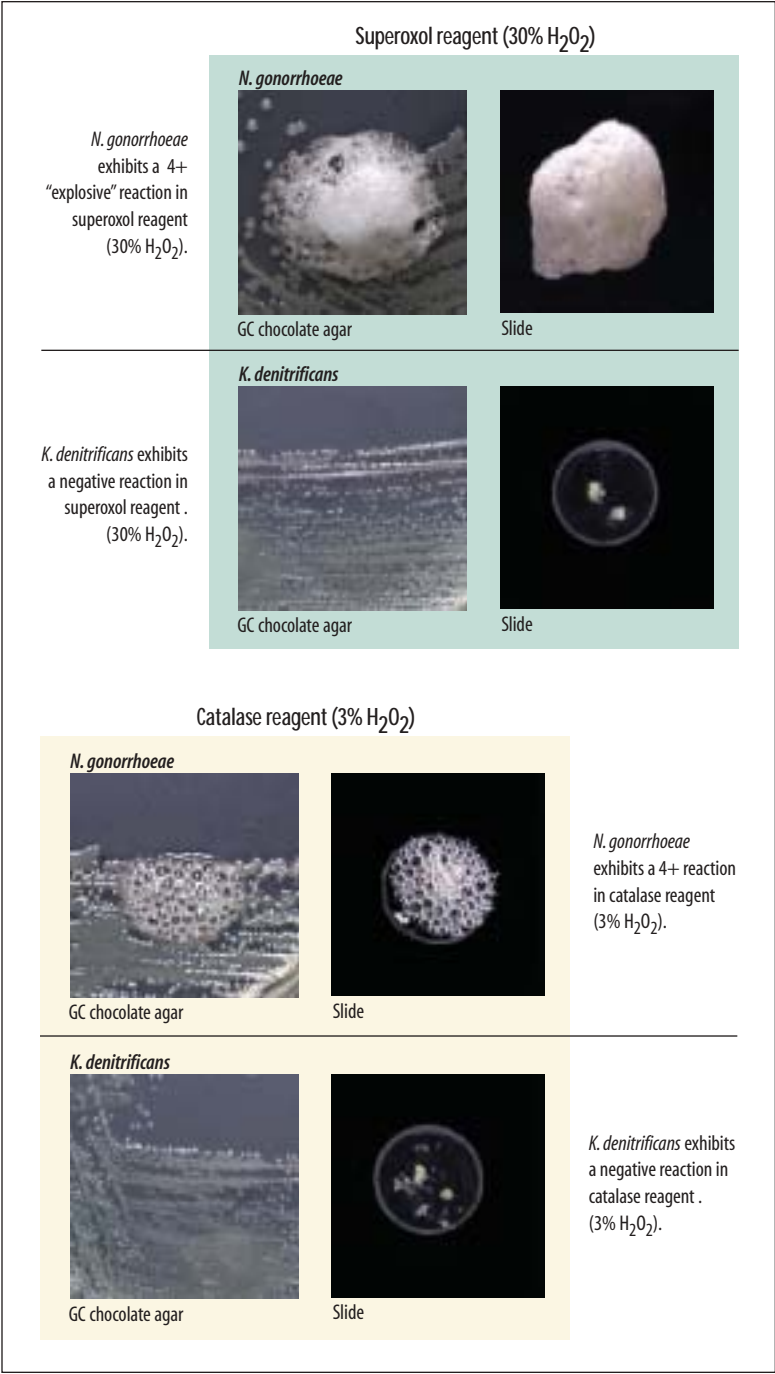
Colistin resistance

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.

²⁰The 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% H₂O₂) and catalase (3% H₂O₂) 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% CO₂ 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 CO₂-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.

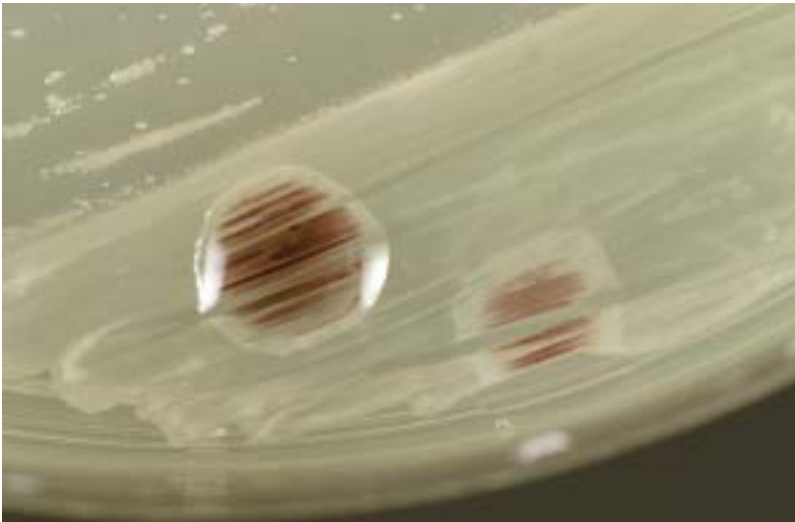
Acid production test

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

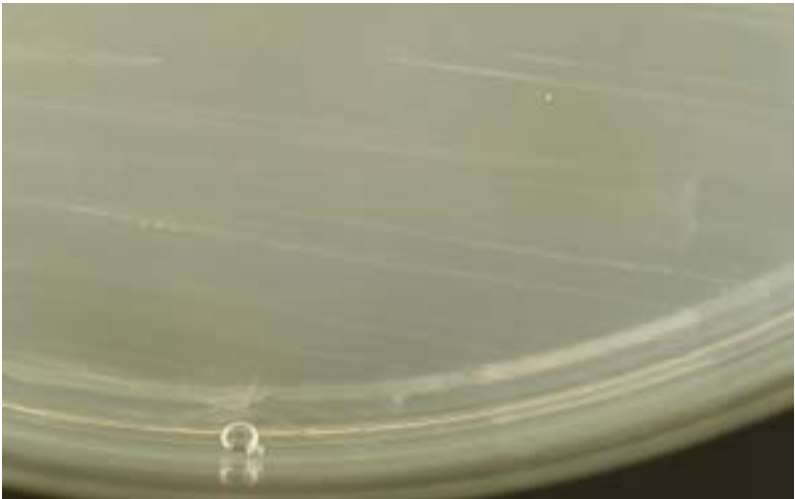
N. polysaccharaea



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 CO₂ 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.

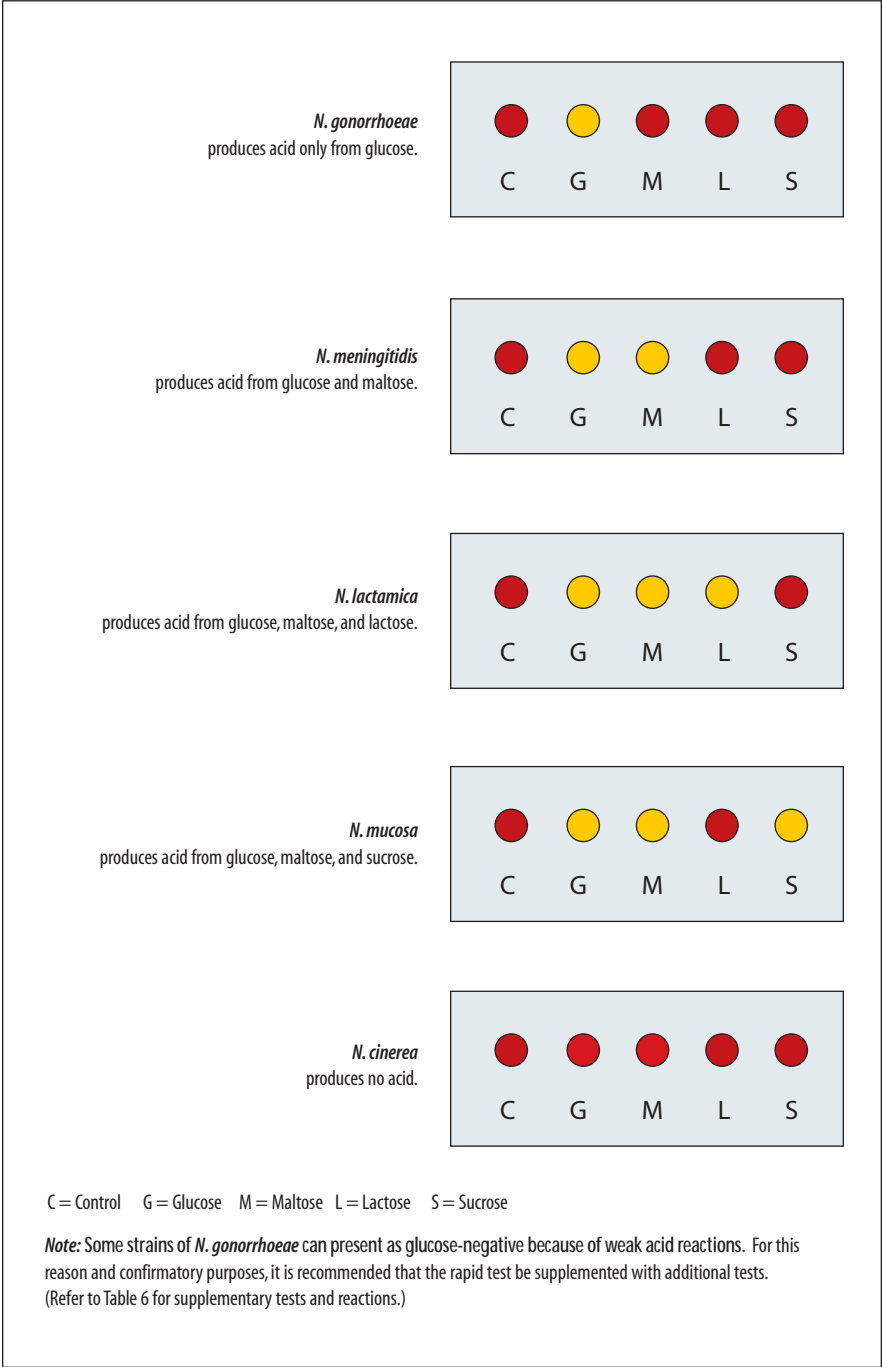
Many of the commercial acid production tests were developed to differentiate among species that routinely grow on selective media for the gonococcus, including *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *M. catarrhalis*. However, interpretive criteria provided with the product (package insert) 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 CO₂ 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 CO₂), 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 (β -galactosidase, γ -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 γ -glutamyl-aminopeptidase), *N. lactamica* (which produces β -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.

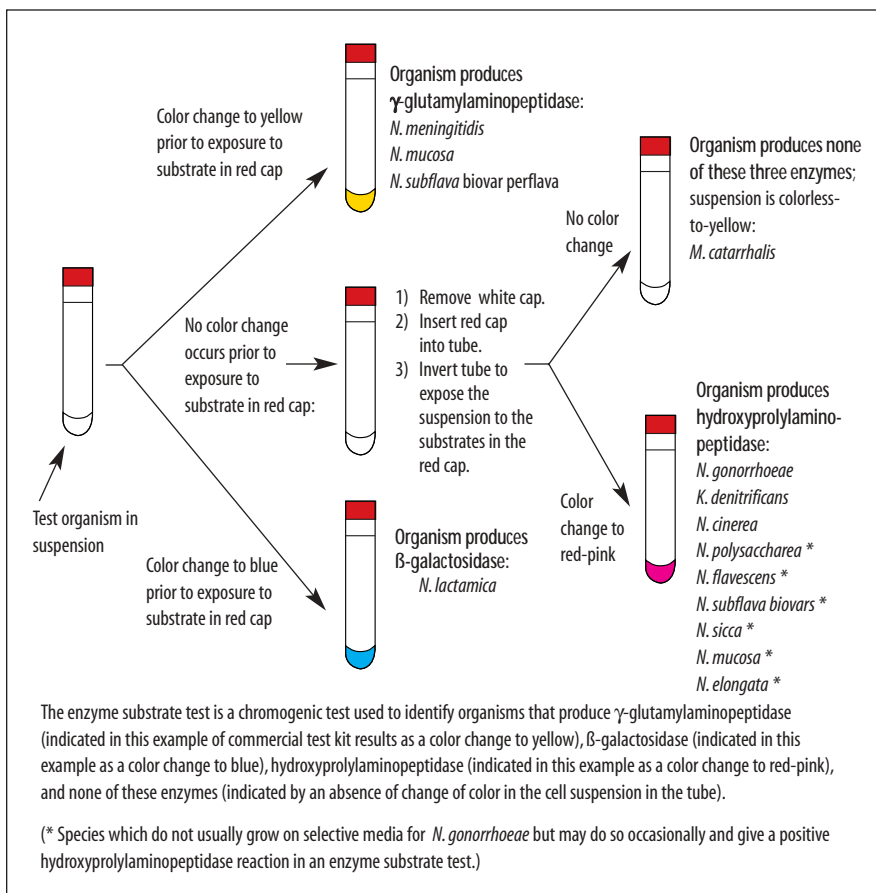
Nitrate reduction test

The nitrate reduction test is available commercially or can be made easily in the laboratory. This test distinguishes between species that can reduce nitrate (NO_3) to nitrite (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"), α -naphthylamine ("Nitrate Reagent B"), and zinc powder ("Zn⁺² 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 α -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 CO₂) 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.

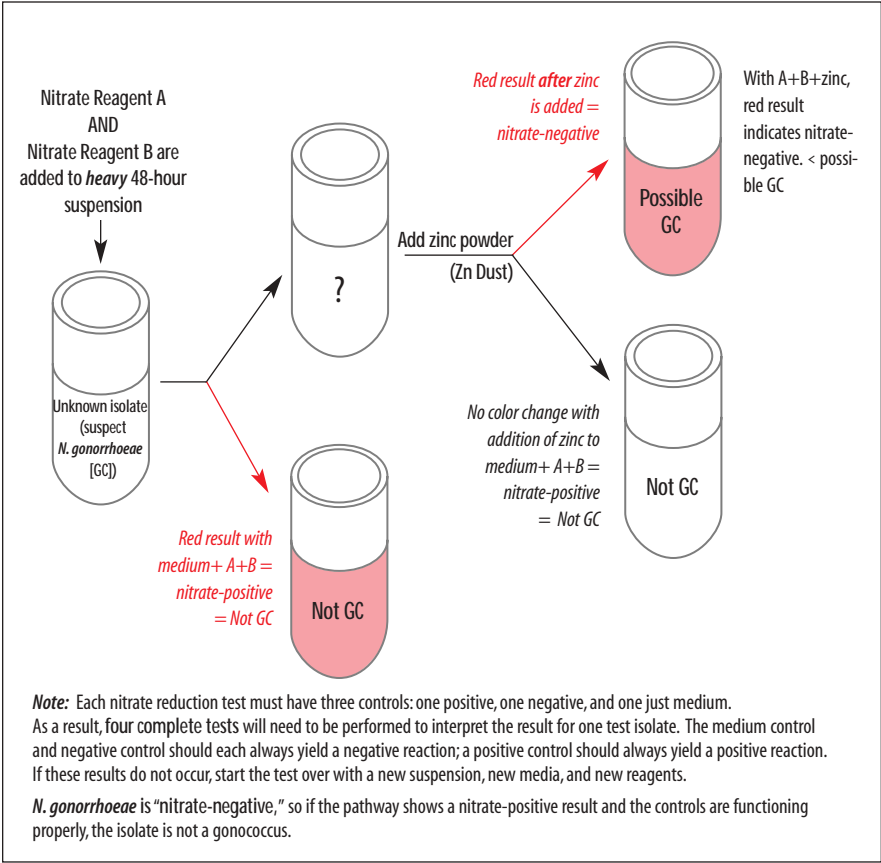
No identification of genus or species can be made on the basis of any of the above biochemical and enzymatic tests alone, but performing a combination (*e.g.*, as presented in Figure 21) can lead to a definitive identification of *N. gonorrhoeae*.

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),²¹ although a variety of methods are used internationally to determine antimicrobial susceptibilities of *N. gonorrhoeae*.

²¹ 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.²²

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.²³ 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

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

²³ 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 ≤ 0.25 mg/ml of cefixime (corresponding disk diffusion zone of inhibition diameter with a 5- μ g cefixime disk, ≥ 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 ≥ 1.0 mg/ml (corresponding disk diffusion zone diameter of inhibition with a 5 mg ciprofloxacin disk, ≤ 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 ≤ 0.06 mg/ml (zone inhibition diameter ≥ 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.²⁴ 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

²⁴ 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.²⁵

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).²⁶ 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

²⁵ 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.

²⁶ 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 β -lactamase (described below). This is because, based on the level of resistance to penicillin and tetracycline and the detection of β -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 β -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 β -lactamase. It should be noted that while plasmid-mediated resistance to penicillin can be detected and confirmed with a simple test to detect β -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	β -lactamase results and specific MIC values associated with phenotype definitions for <i>Neisseria gonorrhoeae</i> ^{a, b}
Susceptible	Isolates exhibiting <i>either</i> susceptibility <i>or</i> intermediate resistance to <i>both</i> penicillin and tetracycline	β -lactamase negative isolate exhibiting: <ul style="list-style-type: none"> • Susceptibility to penicillin [MIC < 2.0 μg/ml (>26 mm)] • Susceptibility to tetracycline [MIC < 2.0 μg/ml (>30 mm)]
PPNG	Penicillinase-producing <i>Neisseria gonorrhoeae</i>	β -lactamase positive isolate. Approximately six β -lactamase plasmids have been identified in <i>N. gonorrhoeae</i> , most commonly: <ul style="list-style-type: none"> • “Asian” = 4.4 megadaltons (Mda) (7.2 kb) • “African” = 3.2 Mda (5.3 kb) • “Toronto” = 3.05 Mda (4.7 kb) (PPNG is defined only by production of β -lactamase and not by MICs of penicillin.) ^c
TRNG	Tetracycline resistant <i>Neisseria gonorrhoeae</i>	β -lactamase negative isolates possessing a TetM-containing conjugative plasmid. TRNG isolates will exhibit both: <ul style="list-style-type: none"> • Susceptibility to penicillin [MIC < 2.0 μg/ml (>26 mm)] • Resistance to tetracycline with MIC \geq 16.0 μg/ml (\leq20 mm) 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>	β -lactamase positive isolates of <i>N. gonorrhoeae</i> exhibiting: <ul style="list-style-type: none"> • Resistance to tetracycline with MIC \geq 16.0 μg/ml (<20 mm)
PenR	Chromosomally mediated resistance to penicillin	β -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> • Resistance to penicillin with MIC \geq 2.0 μg/ml (\leq26 mm) • Susceptibility to tetracycline [MIC < 2.0 μg/ml (>30 mm)]
TetR	Chromosomally mediated resistance to tetracycline	β -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> • Susceptibility to penicillin [MIC < 2.0 μg/ml (>26 mm)] • Resistance to tetracycline with an MIC range of 2.0 μg/ml - 8.0 μg/ml (20 – 30mm)
CMRNG	Chromosomally mediated resistant <i>Neisseria gonorrhoeae</i>	β -lactamase negative isolates exhibiting both: <ul style="list-style-type: none"> • Resistance to penicillin with MIC \geq 2.0 μg/ml (\leq26 mm) • Resistance to tetracycline with MIC \geq 2.0 μg/ml (\leq30 mm)

^a **Note:** Some TRNG may exhibit tetracycline MICs <16.0 μ g/ml, and some TetR isolates may exhibit tetracycline MICs \geq 16.0 μ 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.

^b **Note:** For some research purposes, a breakpoint of 1.0 μ 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].

^c **Note:** For PPNG isolates, MICs for penicillin are typically high (\geq 8.0 μ 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 μ g/ml of penicillin but are still β -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 characterization²⁷ 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 β -lactamase testing, providing the laboratory with phenotypic data appropriate for international comparisons.

Tests identifying gonococcal strains that produce β -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

²⁷ Examples of phenotypic typing include auxotyping (determination of nutrients required for growth of a strain), serotyping, β -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 β -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 β -lactamase production by *N. gonorrhoeae*

The most reliable way to detect β -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 “ β -lactamase positive”. Strains for which the inoculum on the nitrocefin disk does not change color are considered “ β -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 β -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 β -lactamase, and should be recorded as “ β -lactamase positive.” If, after ten minutes, no color change has occurred on the colonies dropped with reagent, the gonococcal strain is considered “ β -lactamase negative,” and should be recorded as such.

To perform the test for β -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 β -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 ‘ β -lactamase positive.’ If after five minutes no color change has occurred in the suspension, record the strain as ‘ β -lactamase negative.’

Results of β -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*

Date of Testing: _____/_____/_____ <i>Test performed by:</i> _____		Interpretation of susceptibility: S = susceptible I = intermediate R = resistant									
Specimen number	Antimicrobial: Ciprofloxacin	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:	Antimicrobial:
	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) ^a											
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
^a Choice of supplemental QC strains will depend upon the antimicrobial agents tested, and therefore several QC strains may be indicated.											
Reviewed by: _____ Date of Report: _____/_____/_____											

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 CO₂-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 swab²⁸ 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.

²⁸ 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 swab²⁸ 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% CO₂ atmosphere (in a CO₂-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 strip²⁹ 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.

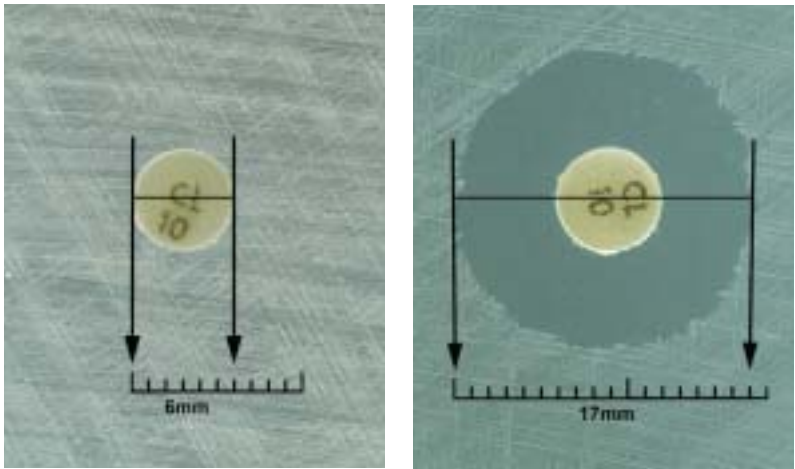
Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on GC base medium plus 1% defined growth supplement; methods for the preparation and QC of this medium are included in Appendix 2 ("Media, Reagents and Quality Control"). The standardization of the inoculum and methods for the inoculation 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.³⁰

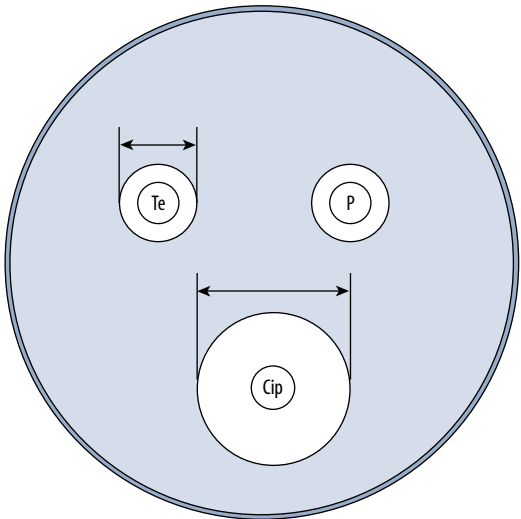
²⁹ The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor settings (see Appendix 13).

³⁰ Please note that for certain antimicrobial agents (particularly some β -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) [µg/ml] ^a							
	Strain ^b	ATCC 49226 ^c	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 µg/ml]	26 – 34 [0.25 – 1.0]	37 – 47 [0.015 – 0.06]	6 – 10 [4.0 – 64.0]	<NT> [32.0 – ≥ 64.0] ^e	<NT> [32.0 – ≥ 64.0] ^e	<NT> [0.5 – 1.0]	<NT> [4.0 – 8.0]
Tetracycline (30-µg)	Disk (mm) [MIC µg/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-µg)	Disk (mm) [MIC µg/ml]	23 – 29 [8.0 – 32.0]	6 – 7 [≥ 128.0]	22 – 25 [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]	<NT> [< 128.0]
Ceftriaxone (30-µg)	Disk (mm) [MIC µg/ml]	39 – 51 [0.004 – 0.016]	49 – 62 [0.0005 – 0.004]	43 – 53 [0.002 – 0.008]	<NT> [≤ 0.002 – 0.008] ^d	<NT> [0.004 – 0.015]	<NT> [0.004 – 0.015]	<NT> [0.06 – 0.125]
Cefixime (5-µg)	Disk (mm) [MIC µg/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-µg)	Disk (mm) [MIC µg/ml]	48 – 58 [0.001 – 0.008]	40 – 55 [≤ 0.002 – 0.008] ^d	45 – 55 [≤ 0.001 – 0.004] ^d	30 – 34 [0.25 – 0.5]	21 – 26 [1.0 – 2.0]	<NT> [0.002 – 0.015]	<NT> [8.0 – 32.0]
Ofloxacin (5-µg)	Disk (mm) [MIC µg/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-µg)	Disk (mm) [MIC µg/ml]	<NT> [0.125 – 0.5] ^c	<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]
β-lactamase production	(+ / -)	-	-	+	+	+	-	-

^a 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.

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

^c 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.

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

^e 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) ^a			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 ^b (≤ 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.

^a 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.

^b 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% CO₂ 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 ^a
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>CfxDS</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 ^b (≤ 25 mm) ^b	'Resistance' ^b (<i>AzmR</i>)	
Spectinomycin	2 g, single dose, intramuscular (IM)	MIC ≥ 128.0 µg/ml (≤ 14 mm)	Resistance (<i>SpCR</i>)	

^a 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.

^b 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 [Handsfield *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

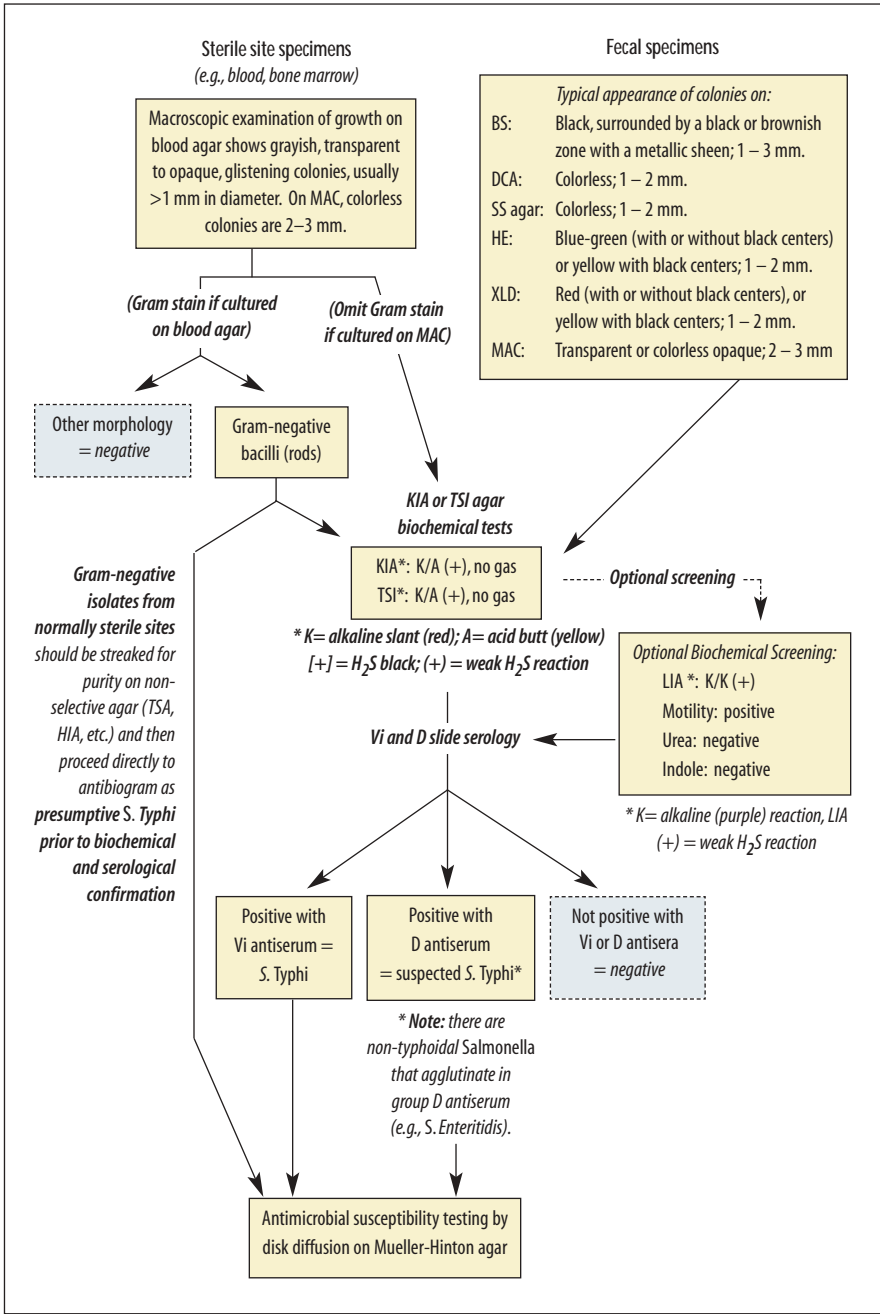


FIGURE 30: Sample worksheet for *Salmonella* ser. Typhi test results

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

^a 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).

^b 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.

^c 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.

^d 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 (H₂S, +) 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 H₂S. *S. Paratyphi* A isolates on TSI/KIA are usually K/AG and do not produce H₂S. Most other *Salmonella* serotypes produce a K/AG+ reaction, indicating that glucose is fermented with gas and H₂S 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 H₂S, 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 H₂S (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(+) ^a	K/AG- ^a	K/AG+ ^a
Kligler iron agar (KIA)	K/A(+) ^a	K/AG- ^a	K/AG+ ^a
Lysine iron agar (LIA)	K/K(+) ^b	K/AG- ^b	K/K+ ^b
Hydrogen sulfide (H ₂ S)	(weak) ^c	negative	positive
Urea	negative	negative	negative
Motility	positive ^c	positive	positive
Indole	negative	negative	negative

^a for KIA / TSI: K = alkaline (red); A = acid (yellow); G = gas production; + = black H₂S produced (weak); – = no H₂S
^b for LIA: K = alkaline (purple); A = acid (yellow); G = gas production; + = black H₂S produced (weak); – = no H₂S
 ~ 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.
^c 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 H₂S 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.

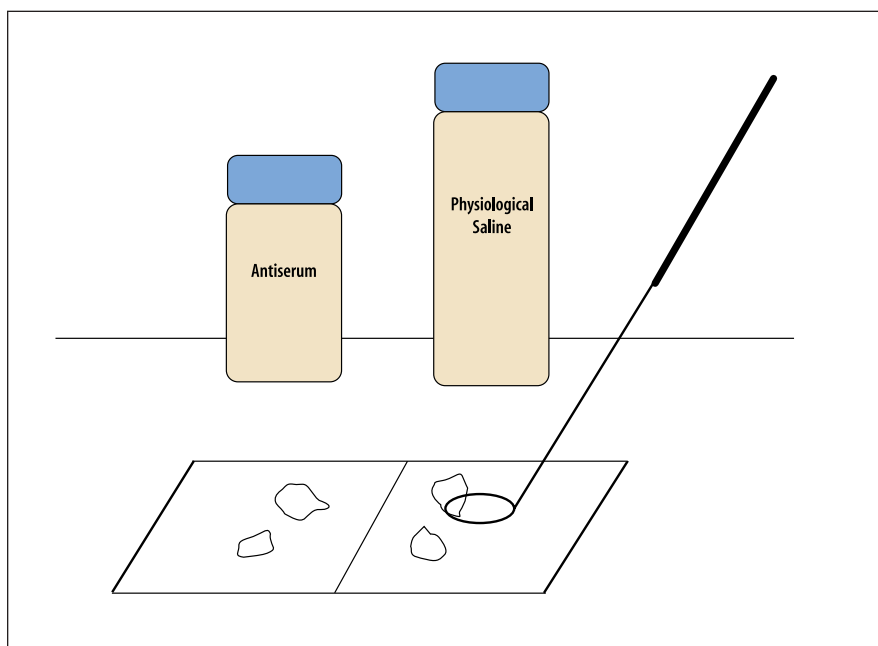
- a) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, LIA, or other nonselective agar medium. Serologic testing should not be done on growth from selective media (e.g., MAC, DCA, BS, or XLD) because selective media 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),³¹ 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].

³¹ 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*

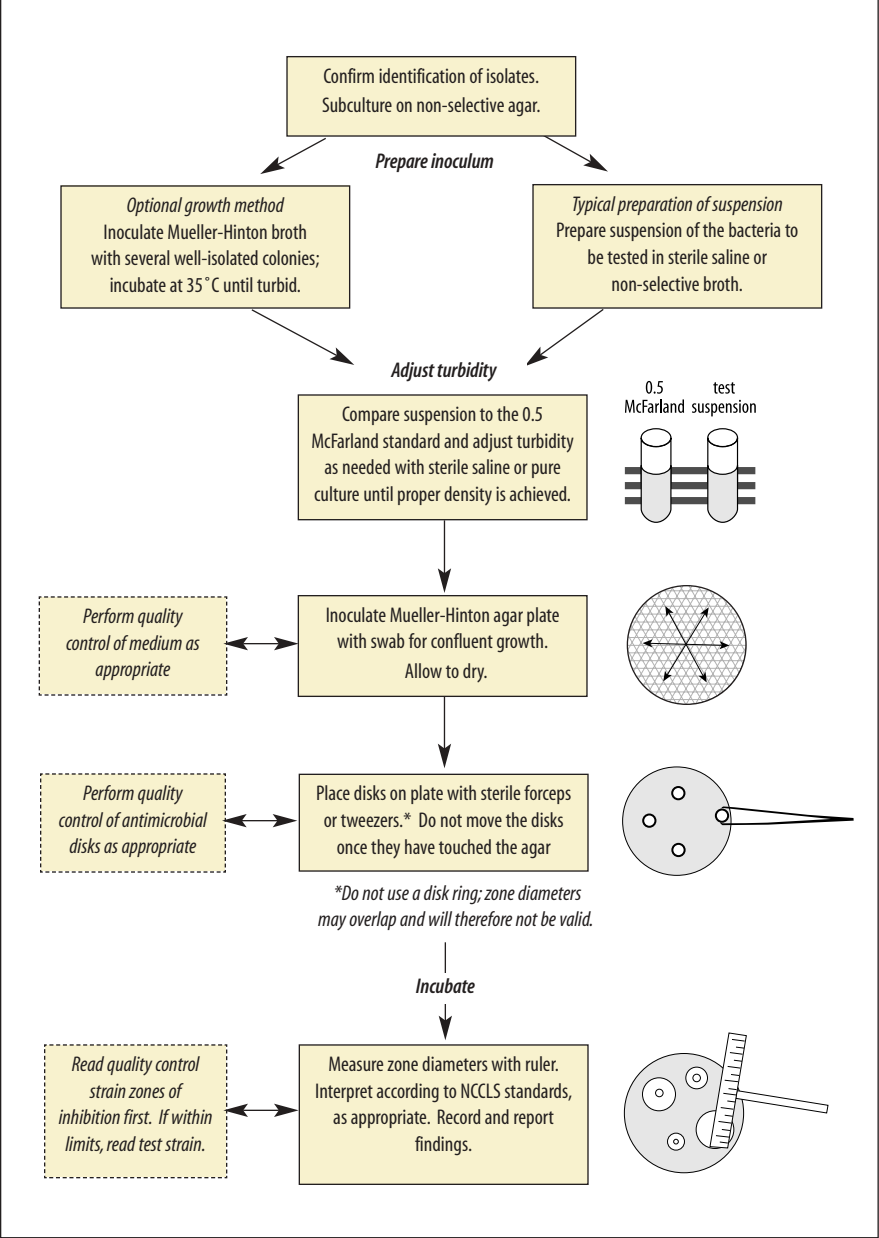
Mueller-Hinton agar medium is the only antimicrobial susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing according to NCCLS and international guidelines. Because the way in which Mueller-Hinton is prepared can 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.

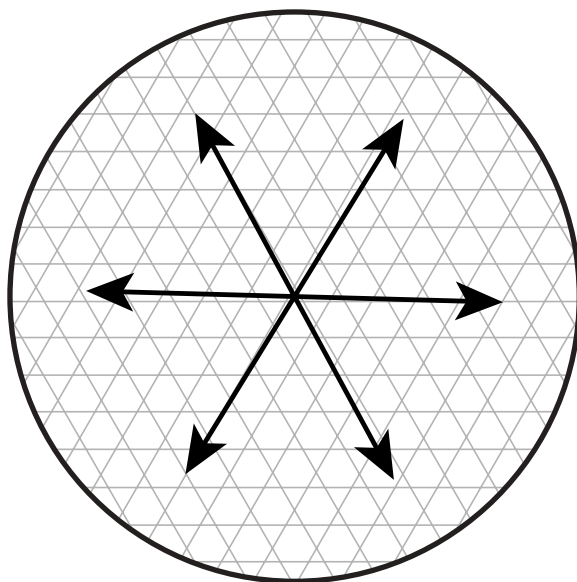
If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture, as may occur when working with cultures from stool specimens), laboratorians may choose to prepare a purity 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).

- 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 H₂S (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*

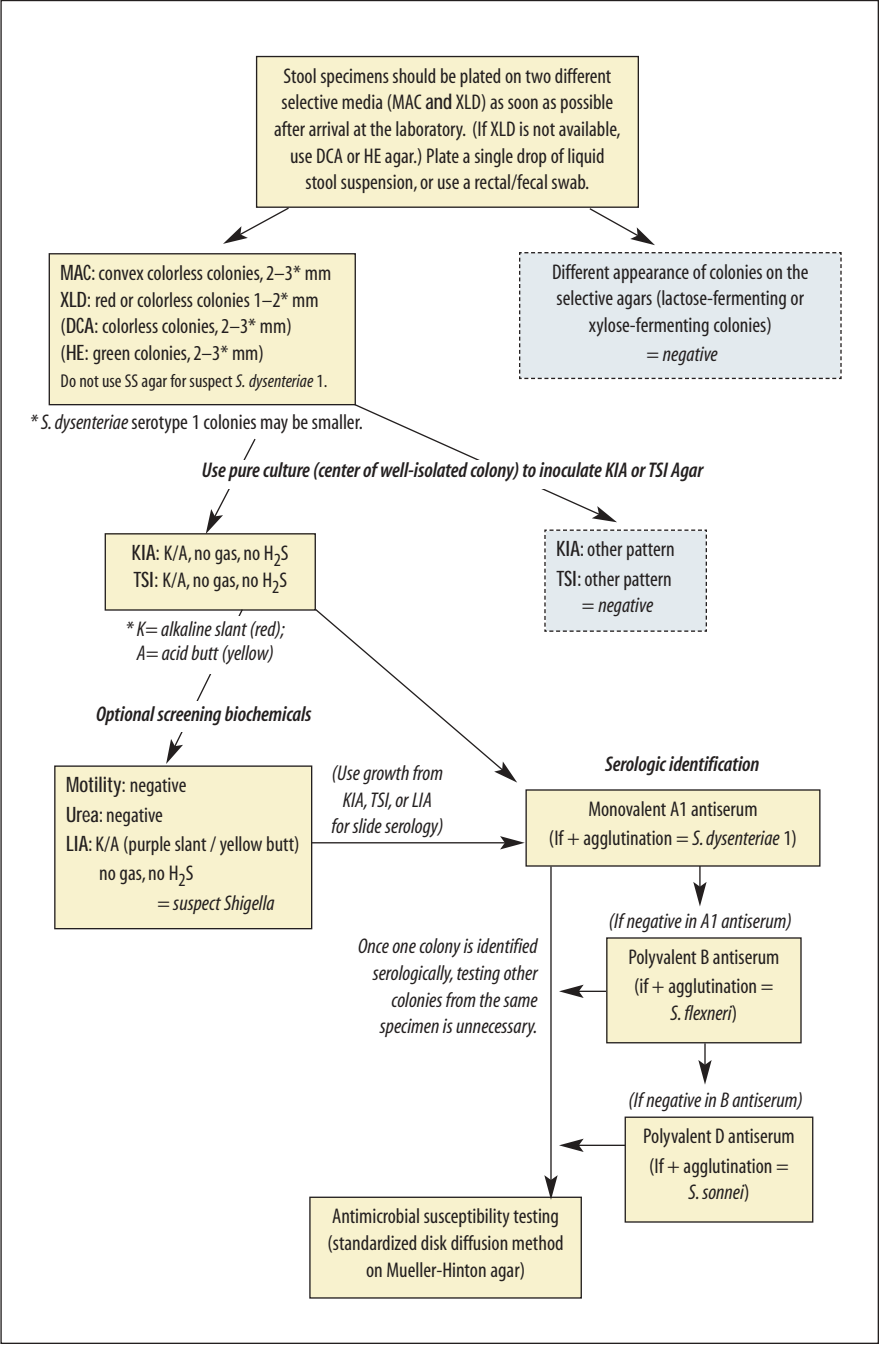


FIGURE 37: Sample worksheet for *Shigella* test results

Specimen number	Medium	XYL/ LAC- ^a	XYL/ LAC+ ^a	Colony ^b	KIA / TSI	OPTIONAL			SLIDE SEROLOGY ^c			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								

^a XYL/LAC-: Xylose or Lactose -negative colonies
XYL/LAC+ : Xylose or Lactose -positive colonies

^b Identification of only one colony from each suspect case must be confirmed as *Shigella*.

^c 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) ^a	Figure 38
Triple sugar iron agar (TSI)	K/A, no gas produced (red slant/yellow butt) ^a	~
H ₂ 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) ^b	Figure 41

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

^b 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).

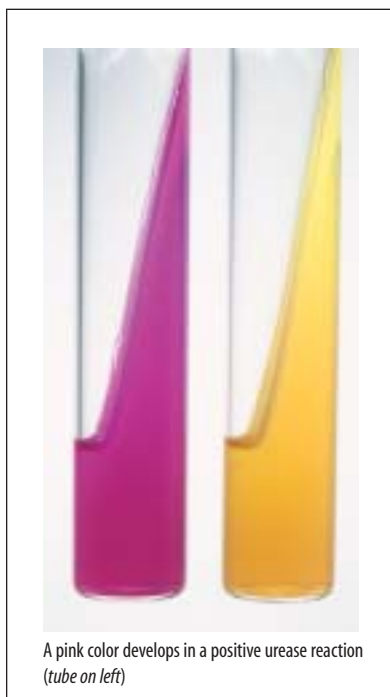
FIGURE 38: Reaction typical of *Shigella* in Kligler iron agar (KIA): alkaline slant and acid butt



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 H₂S production. Organisms lacking lysine decarboxylase produce an alkaline slant (purple) and an acid butt (yellow), no gas, and no H₂S. *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 H₂S 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 ^{a,b}	(A1, A2, A3, . . . , A13)
<i>S. flexneri</i>	Group B	1 – 6, X, Y	(B1, B2, B3, B4, B5, B6)
<i>S. boydii</i> ^c	Group C	1 – 19 ^b	(C1, C2, C3, . . . , C18)
<i>S. sonnei</i>	Group D	1	(D1)

^a 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.

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

^c 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*



Shigella antiserum will agglutinate strains of the same subgroup or serotype (right); in contrast, the *Shigella* suspension on the left did not agglutinate when mixed with saline.

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),³² 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.

³² 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 ^a	(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) QC in range? →	S I R mm Yes No	S I R mm Yes No	S I R mm Yes No	S I R mm Yes No	S I R mm Yes No

^a 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*

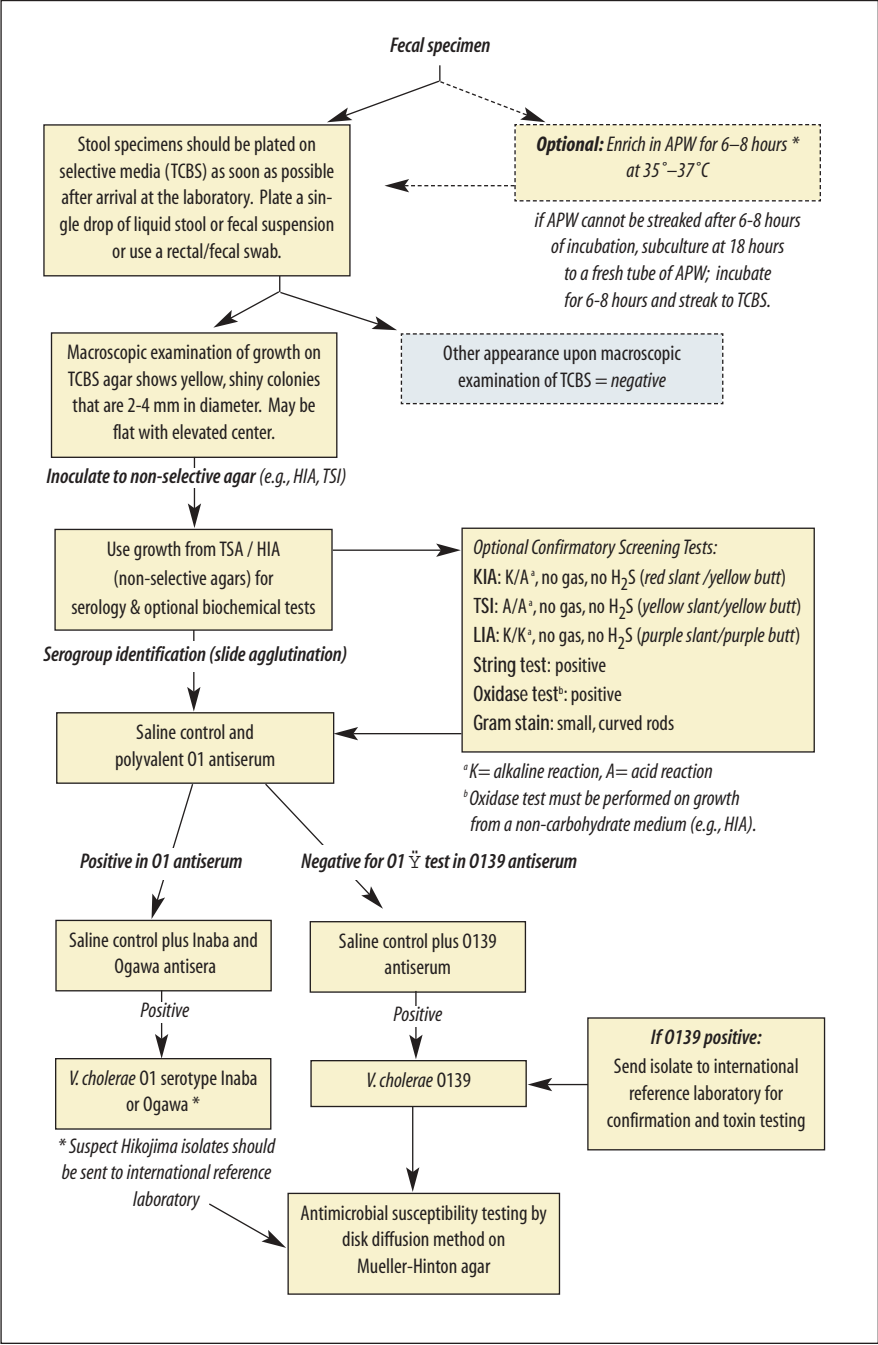


FIGURE 46. Sample worksheet for *Vibrio cholerae* test results

Specimen number	Medium	SUC- ^{a,b}	SUC- ^c	Colony	OPTIONAL			SLIDE SEROLOGY ^d				Identification	
					Oxidase test	String test	Gram stain or wet mount	PV 01	Inaba	Ogawa	O139		
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS ^a			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS ^a			AT1									
				AT2									
				AT3									
	Direct TCBS			T1									
				T2									
				T3									
	APW- TCBS ^a			AT1									
				AT2									
				AT3									

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

^a APW-TCBS :alkaline peptone water enrichment prior to inoculating TCBS

^b SUC + : Sucrose-positive colonies

^c SUC - : Sucrose-negative colonies

^d PV01 = polyvalent antiserum for *V. cholerae* serogroup O1
Inaba= monovalent antiserum for *V. cholerae* O1 serotype Inaba
Ogawa= monovalent antiserum for *V. cholerae* O1 serotype Ogawa
O139 = monovalent antiserum for *V. cholerae* serogroup O139

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) ^a , no gas produced, no H ₂ S [18–24 hours]	Figure 48
Triple sugar iron agar (TSI)	A/A (yellow slant/yellow butt) ^a , no gas produced, no H ₂ S [18–24 hours]	Figure 48
Lysine iron agar (LIA)	K/K (purple slant / purple butt) ^{a,b} , no gas produced, no H ₂ S [18–24 hours]	–
Gram stain	Small, gram-negative curved rods	–
Wet mount	Small, curved rods with darting motility	–

^aK= alkaline; A= acid

^b 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.

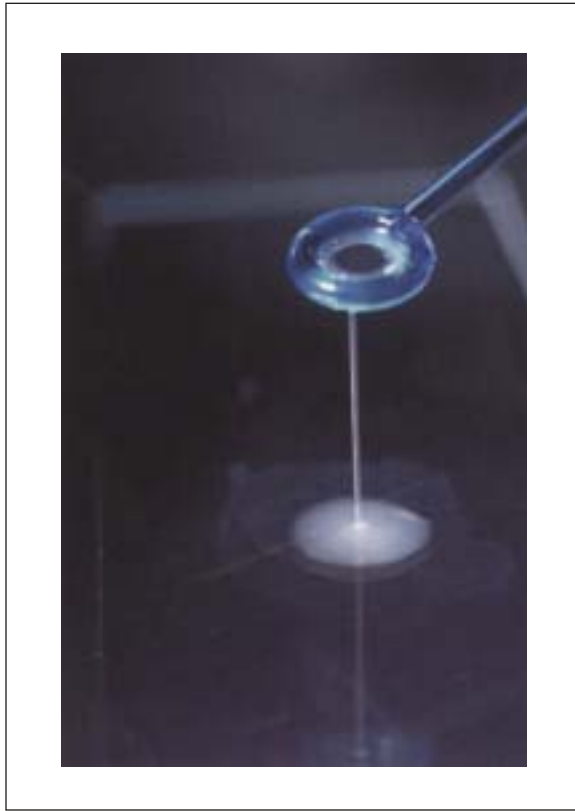
Kligler iron agar and triple sugar iron agar

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*



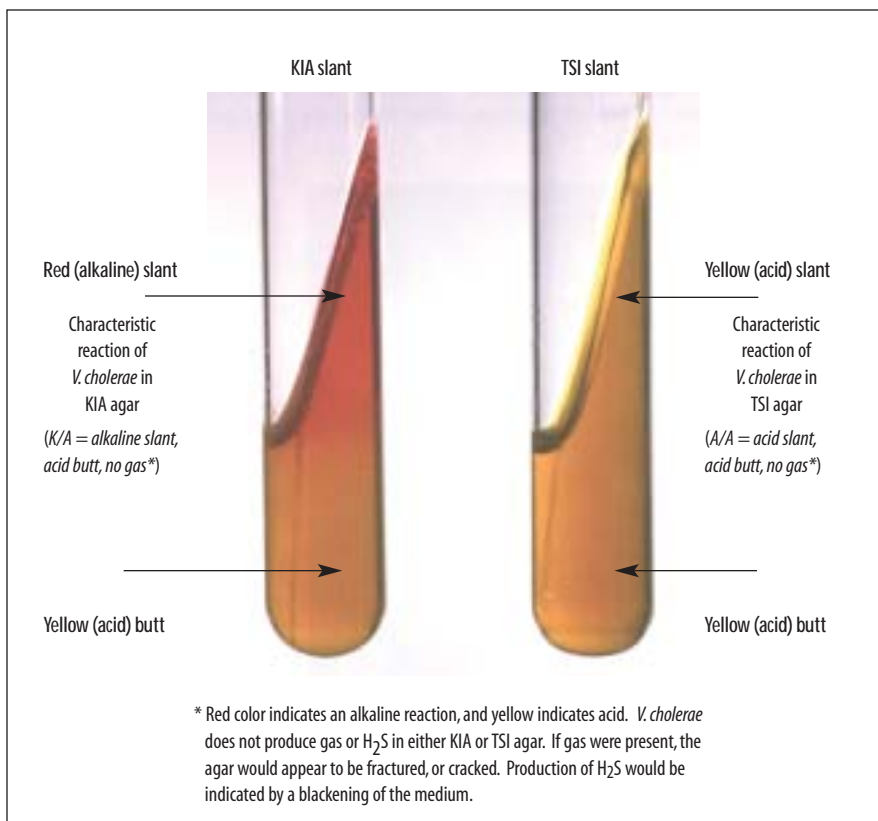
and anaerobic conditions exist in the tube, an inappropriate reaction will occur and the characteristic reactions of *V. cholerae* may not be exhibited.

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 H₂S). However, on TSI, *V. cholerae* strains produce an acid (yellow) slant, acid (yellow) butt, no gas, and no H₂S (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. H₂S 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 H₂S (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	+ ^a	– ^b
Inaba	–	+
Hikojima ^c	+	+

^a + indicates a positive agglutination reaction in the absorbed antiserum.

^b – indicates a negative agglutination reaction in the absorbed antiserum.

^c 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),³³ 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

³³ 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 ^a	
Nalidixic acid ^b	
^a The results from the tetracycline disk are also used to predict susceptibility to doxycycline.	
^b 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 ^a	Trimethoprim-sulfamethoxazole	Tetracycline ^b	Nalidixic acid ^a	(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? →	Yes No mm	Yes No mm	Yes No mm	Yes No mm	Yes No mm

^a 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.

^b 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 ^a	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 ^{a,b}	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 ^c	100 µg	≥ 18 mm	-	< 18 mm	22 – 26 mm ^d -
Nalidixic acid ^c	30 µg	≥ 19 mm	-	< 19 mm	22 – 28 mm (1–4 µg/ml)
Ciprofloxacin ^e	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 ^a	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 ^a (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)

^a 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.

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

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

^d 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.

^e 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.