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

Neisseria gonorrhoeae

CHAPTER VI

Neisseria gonorrhoeae

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

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

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

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

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

Presumptive identification of *N. gonorrhoeae*

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

Because *N. gonorrhoeae* is highly susceptible to adverse environmental conditions (as described in Table 28 of Appendix 8), strains must always be incubated at 35˚–36.5˚C in a humid, CO_2-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

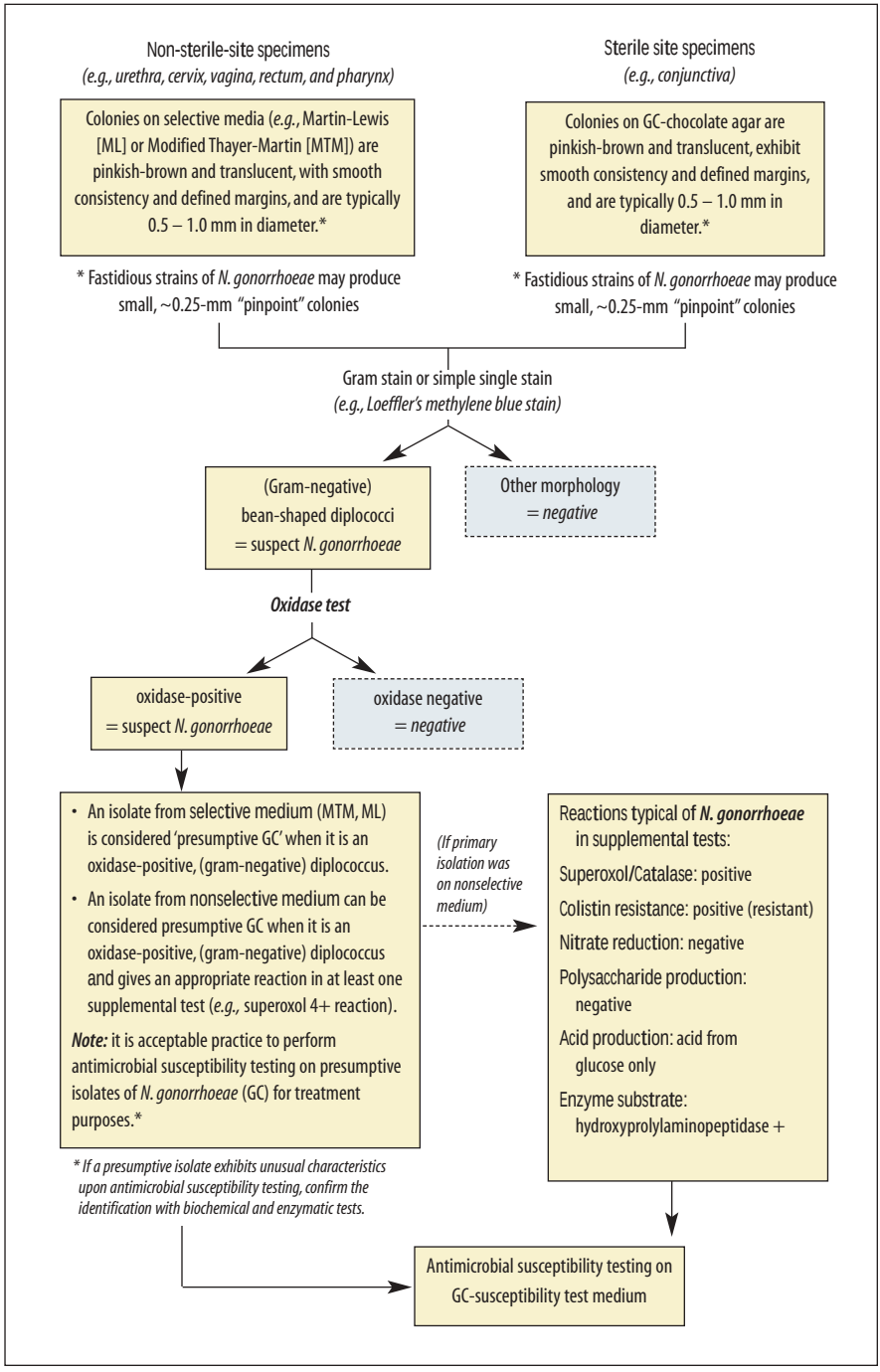
The oxidase test uses Kovac's reagent (a 1% (wt/vol) solution of *N, N, N', N'* –tetramethyl-ρ-phenylenediamine dihydrochloride)[18] to detect the presence of cytochrome c in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. *Neisseria* species give a positive 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-ρ-phenylenediamene dihydrocholoride; "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.

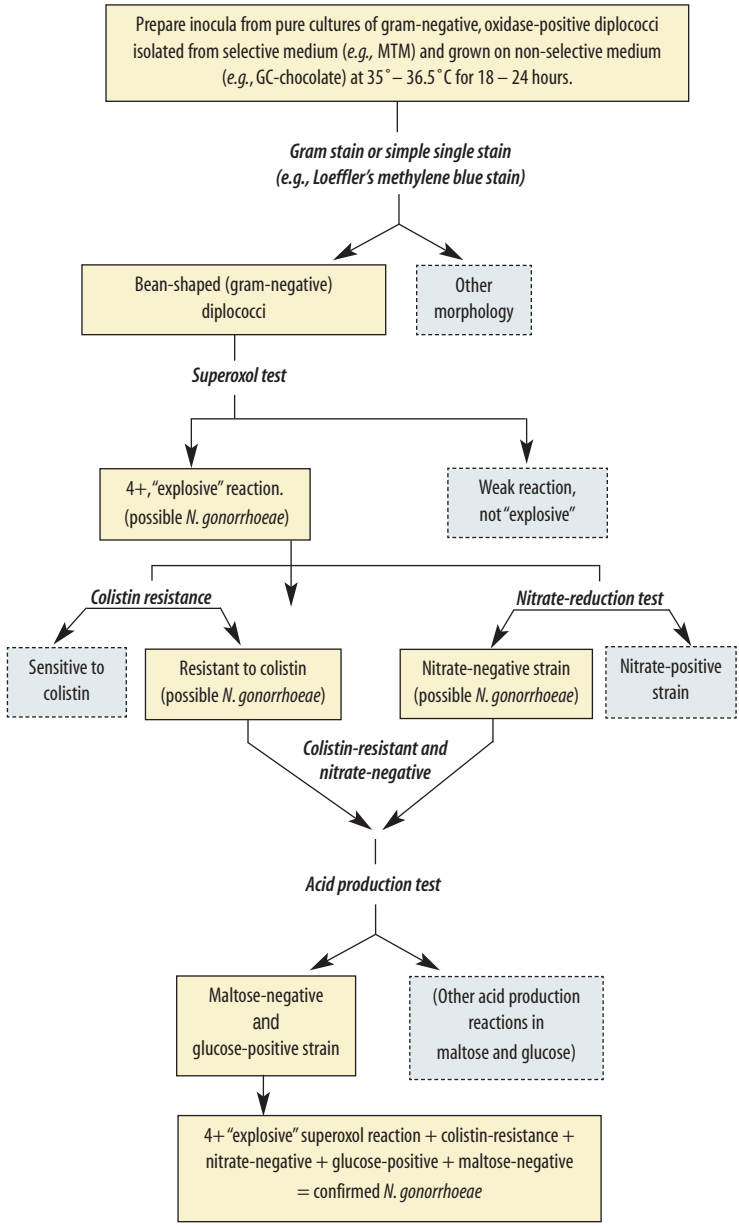
Laboratorians wishing to learn more about the concepts behind the biochemical and enzyme substrate test reactions presented here, or seeking information about other tests and methodologies in more detail, can refer to the American Society of Microbiology's *Manual of Clinical Microbiology,* or, for example, 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***



Note: if resources are available, several confirmatory tests may be run concurrently, rather than waiting for results from each test before continuing.

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

Species	Cell morphology	Superoxol *{Catalase}*	Colistin	Production of acid from: GLU	MAL	LAC	SUC	Reduction of NO_3 (Nitrate)	Polysaccharide from sucrose
Test isolate:									
N. gonorrhoeae [a]	GND	4+ *{+}*	R	+	–	–	–	–	–
N. meningitidis	GND	1+ to 4+ *{+}*	R	+	+	–	–	–	–
N. lactamica	GND	1+ to 3+ *{+}*	R	+	+	+	–	–	–
N. cinerea [b]	GND	2+ *{+}*	(R)	–	–	–	–	–	–
N. polysaccharea	GND	1+ to 3+ *{+}*	(R)	+	+	–	–	–	–
N. subflava [b]	GND	2+ *{+}*	(R)	+	+	–	V	–	V
N. sicca	GND	2+ *{+}*	S	+	+	–	+	–	+
N. mucosa	GND	2+ *{+}*	S	+	+	–	+	+	+
N. flavescens	GND	2+ *{+}*	S	–	–	–	–	–	+
N. elongata	GNR	– *{–}*	S	–	–	–	–	–	–
M. catarrhalis	GND	1+ to 4+ *{+}*	(R)	–	–	–	–	+	–
K. denitrificans [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	*N. gonorrhoeae* ATCC 49226 [4+] *N. cinerea* ATCC 14685 [weak, 2+] (positive reaction in superoxol)	*K. denitrificans* ATCC 33394 (no reaction in superoxol)
Colistin resistance test	*N. gonorrhoeae* ATCC 49226 *K. denitrificans* ATCC 33394 (resistant to colistin)	*N. cinerea* ATCC 14685 *N. mucosa* ATCC 19696 (susceptible to colistin)
Polysaccharide production test	*N. polysaccharea* ATCC 43768 *N. mucosa* ATCC 19696 (produce polysaccharide)	*N. gonorrhoeae* ATCC 49226 *N. cinerea* ATCC 14685 (do not produce polysaccharide)
Nitrate reduction test	*K. denitrificans* ATCC 33394 *N. mucosa* ATCC 19696 (able to reduce nitrate)	*N. gonorrhoeae* ATCC 49226 *N. cinerea* ATCC 14685 (unable to reduce nitrate)
Acid production test	Use the QC strains recommended by the test manufacturer* plus *N. cinerea*. * If the manufacturer has not designated specific strains for QC: • *N. gonorrhoeae* (ATCC 49226) produces acid from glucose • *N. meningitidis* (ATCC 13077) produces acid from glucose and maltose • *N. lactamica* (ATCC 23970) produces acid from glucose, maltose, and lactose • *N. mucosa* (ATCC 19696) produces acid from glucose, maltose, and sucrose • *N. cinerea* (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: • *N. gonorrhoeae* (ATCC 49226) produces hydroxyprolylaminopeptidase. • *N. meningitidis* (ATCC 13077) produces γ-glutamylaminopeptidase. • *N. lactamica* (ATCC 23970) produces ß-galactosidase. • *M. catarrhalis* (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.

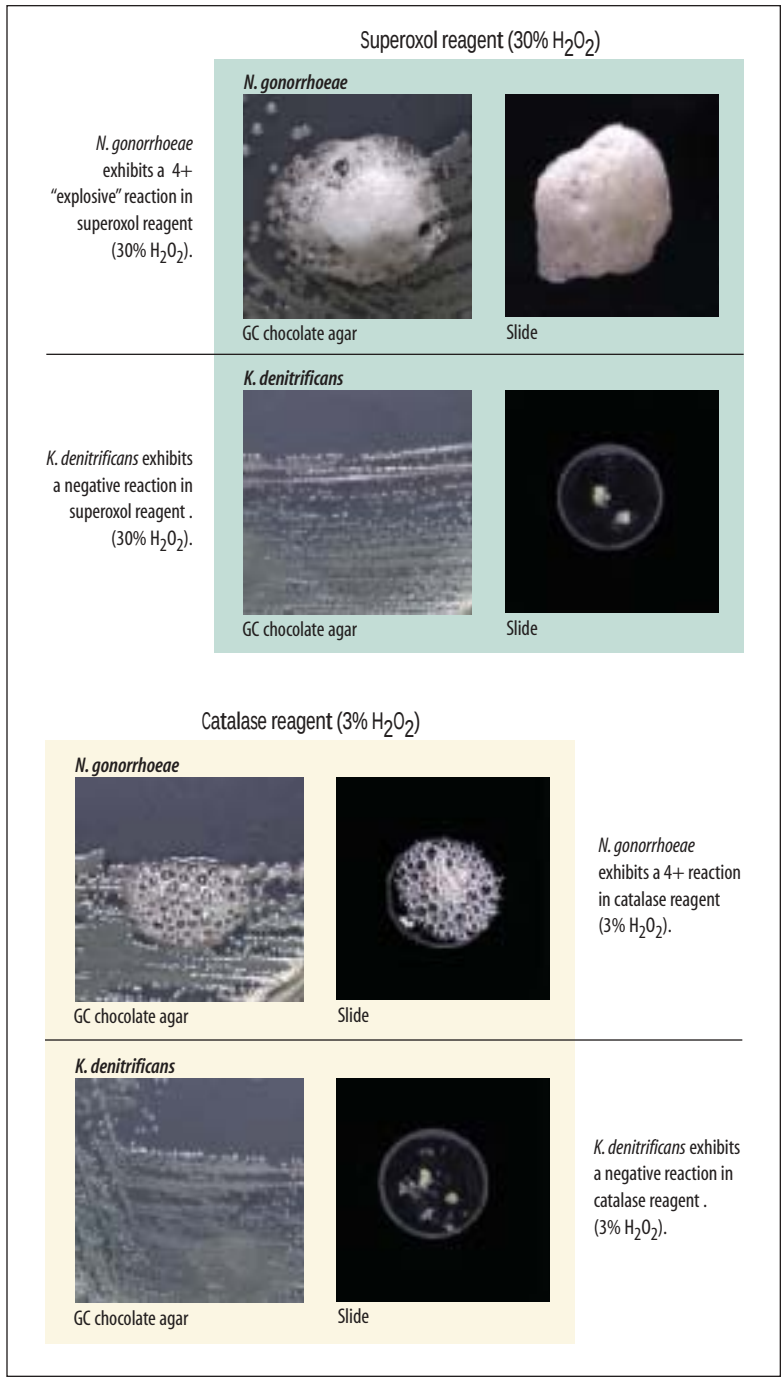
Colistin resistance

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.

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

FIGURE 22: Positive and negative reactions in superoxol (30% H_2O_2) and catalase (3% H_2O_2) reagents



- A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the colistin resistance test at once, **and the colistin disks are from the same batch,** it is appropriate to run the positive and negative controls on only one plate.

b) Prepare a suspension of a pure overnight culture (approximately equal to a 0.5 McFarland turbidity standard) in Mueller-Hinton broth or phosphate buffered saline (PBS).

c) Using a sterile swab or inoculating loop, inoculate the GC-chocolate agar plate evenly with a swab. Allow the plate to dry so that there is no visible surface moisture.

d) Apply a colistin disk (10 μg) to the center of the plate, tapping it down to ensure even contact with the surface. Incubate at 35˚–36.5˚C in 5% CO_2 and increased humidity for 18–24 hours.

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

Polysaccharide production test

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

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

- A 100-mm plate can be divided into four sectors, permitting testing of two clinical strains alongside the positive and negative controls. If there are multiple clinical strains requiring the polysaccharide test at once, **and the**

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

b) Use a sterile swab or loop to inoculate the polysaccharide test medium with pure culture.
   - Although this test is best performed on isolated colonies, because *N. gonorrhoeae* and strains of some other species do not grow well on this medium, the plate should be inoculated heavily for confluent growth so that the test can detect starch produced by pre-formed enzyme in the inoculum itself.

c) Incubate medium at 35˚–36.5˚C in a CO_2-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.*

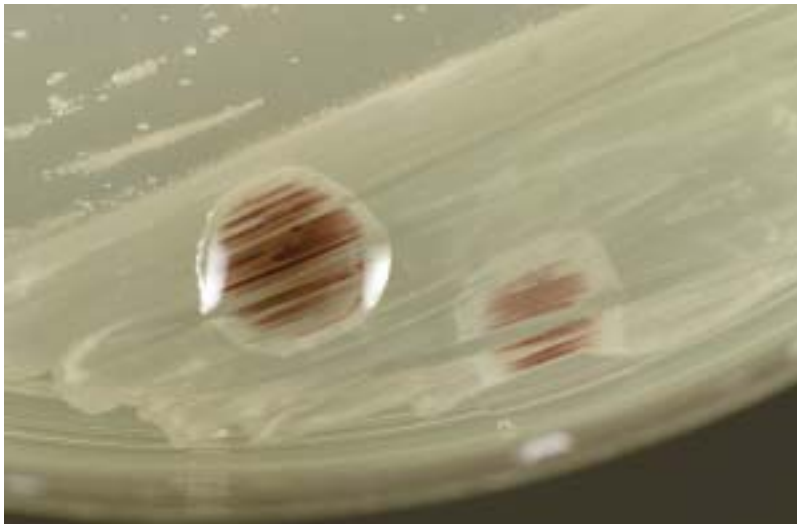
Quality control should be performed with each new batch of sucrose medium or reagent. **This is particularly important because some commercial preparations of Gram's iodine will not react with the starch, yielding false-negative results.** Examples of controls for the polysaccharide-production test are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

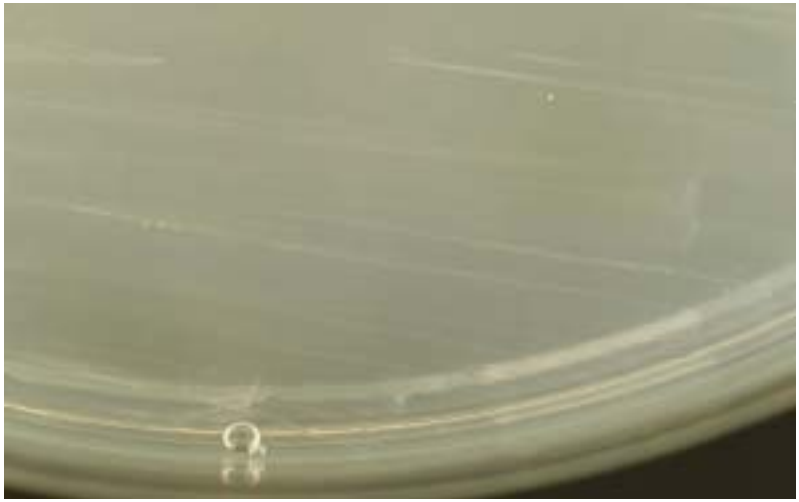
FIGURE 23: Positive and negative results of the polysaccharide-production test on sucrose medium

Polysaccharide postitive *N. polysaccharea*



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

Polysaccharide negative *N. gonorrhoeae*



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

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

Because CTA-sugar media can exhibit misleading results for some strains of *N. gonorrhoeae*, as described above, this laboratory manual advises that, if available, a commercial test be used if it is necessary to detect acid production to confirm the identification of an isolate as *N. gonorrhoeae*. Perform the test according to the manufacturer's instructions and using the manufacturer's recommendations for quality control; note that incubation of the acid production test must occur in an atmosphere **without** supplemental CO_2 in order to avoid false-positive results. It is important that the test chosen to detect acid production be able to differentiate between *N. gonorrhoeae* and *N. cinerea* and *M. catarrhalis*. Reaction patterns of various *Neisseria* species in the acid production test are illustrated in Figure 24.

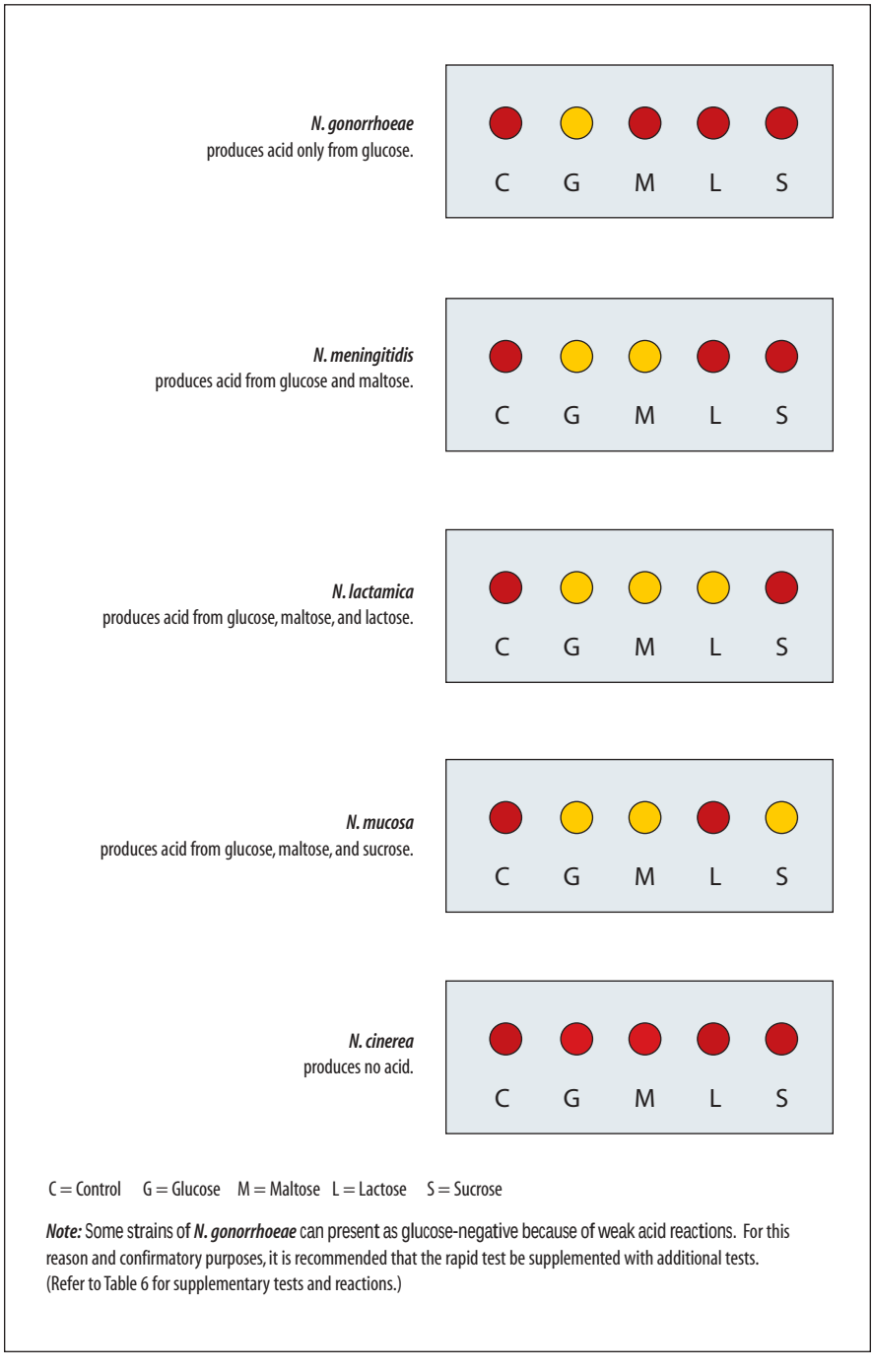
Many of the commercial acid production tests were developed to differentiate among species that routinely grow on selective media for the gonococcus, including *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *M. catarrhalis*. However, interpretive criteria provided with the product (package insert)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

It is suggested that a strain of *N. cinerea* be included among QC strains for the acid production test (in addition to *N. gonorrhoeae* and others). Although *N. cinerea* is considered to be glucose-negative and will be listed as such in tables of acid production reactions, it does actually produce acid from glucose and then rapidly over-oxidize it to produce CO_2 and water; as a result, it may either appear negative or give a weak positive glucose reaction (due to residual acid produced from the glucose and not over-oxidized, and/or due to residual carbonic acid from the production of CO_2), 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 (ß-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 Gonochek-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



Note: Some strains of ***N. gonorrhoeae*** can present as glucose-negative because of weak acid reactions. For this reason and confirmatory purposes, it is recommended that the rapid test be supplemented with additional tests. (Refer to Table 6 for supplementary tests and reactions.)

usually limited to distinguishing between *N. gonorrhoeae* (which produces only hydroxyprolylaminopeptidase), *N. meningitidis* (which produces γ-glutamyl-aminopeptidase), *N. lactamica* (which produces ß-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.

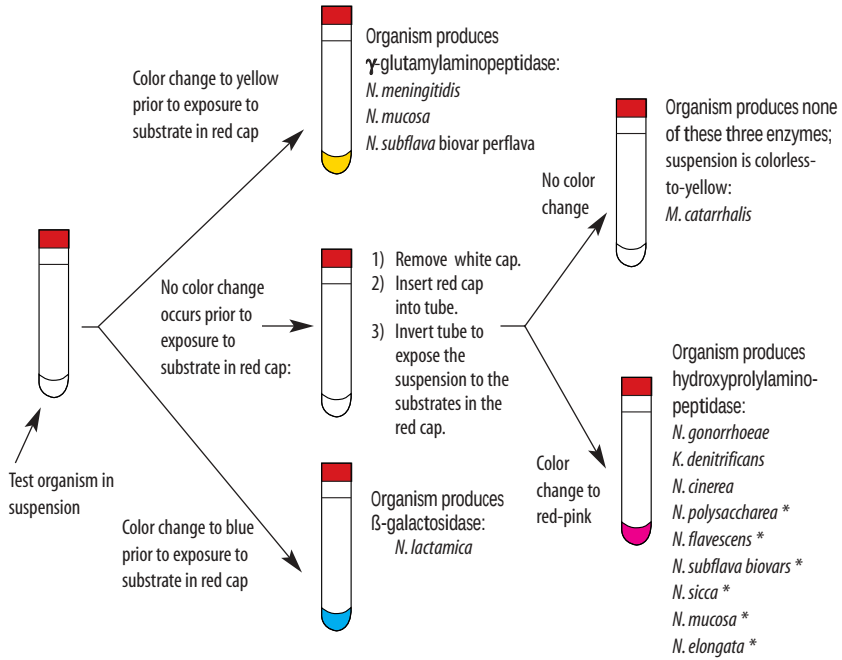
Nitrate reduction test

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



The enzyme substrate test is a chromogenic test used to identify organisms that produce γ-glutamylaminopeptidase (indicated in this example of commercial test kit results as a color change to yellow), ß-galactosidase (indicated in this example as a color change to blue), hydroxyprolylaminopeptidase (indicated in this example as a color change to red-pink), and none of these enzymes (indicated by an absence of change of color in the cell suspension in the tube).

(* Species which do not usually grow on selective media for *N. gonorrhoeae* but may do so occasionally and give a positive hydroxyprolylaminopeptidase reaction in an enzyme substrate test.)

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

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

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

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

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

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

Methods

a) Using colonies from a fresh, pure culture on GC-chocolate agar, prepare a heavy suspension in nitrate broth.

b) Remove the screw-cap top from the tube of nitrate test medium and inoculate the medium to give heavy turbidity. Replace the screw-cap top.

c) Incubate the inoculated tubes and an uninoculated medium control tube at 35˚–36.5˚C (without supplemental CO_2) 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.

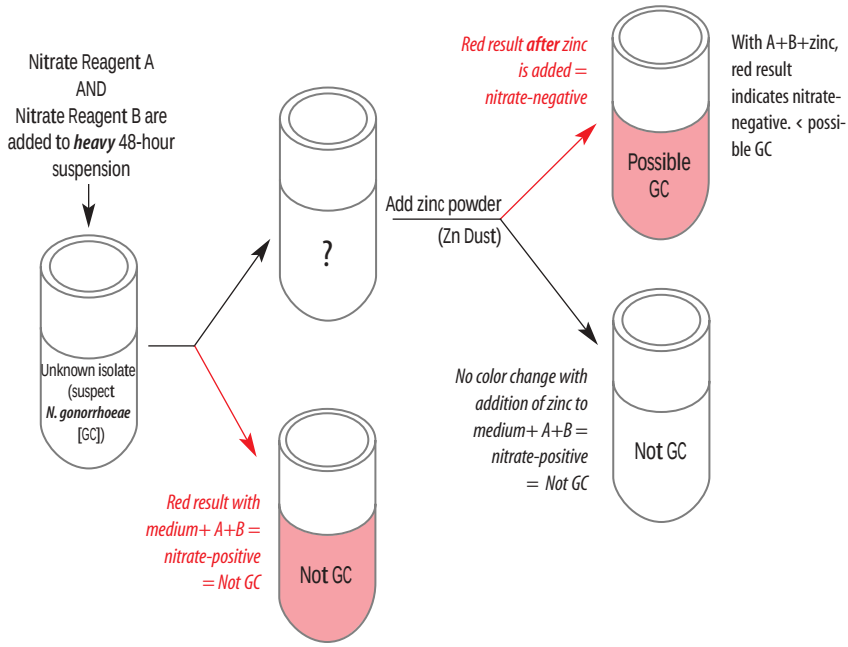
No identification of genus or species can be made on the basis of any of the above biochemical and enzymatic tests alone, but performing a combination (*e.g.*, as presented in Figure 21) can lead to a definitive identification of *N. gonorrhoeae.*

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



Note: Each nitrate reduction test must have three controls: one positive, one negative, and one just medium. As a result, four complete tests will need to be performed to interpret the result for one test isolate. The medium control and negative control should each always yield a negative reaction; a positive control should always yield a positive reaction. If these results do not occur, start the test over with a new suspension, new media, and new reagents.

N. gonorrhoeae is "nitrate-negative," so if the pathway shows a nitrate-positive result and the controls are functioning properly, the isolate is not a gonococcus.

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 (±) one dilution. For example, an organism with an MIC of penicillin of 0.25 μg/ml may exhibit an MIC of 0.125 μg/ml to 0.5 μg/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 μg/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-μg cefixime disk, ≥ 31mm). 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, ≤ 27mm) 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 μg/ml – 0.5 μg/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 treat gonorrhea) and by a simple test to detect the production of ß-lactamase (described below). This is because, based on the level of resistance to penicillin and tetracycline and the detection of ß-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 ß-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 ≥ 2.0 mg/ml, or equivalent inhibition zone diameter ≤ 26 mm) **and** tetracycline (MIC ≥ 2.0 mg/ml, or equivalent inhibition zone diameter ≤ 30 mm) **and** do not produce ß-lactamase. It should be noted that while plasmid-mediated resistance to penicillin can be detected and confirmed with a simple test to detect ß-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	ß-lactamase results and specific MIC values associated with phenotype definitions for ***Neisserea gonorrhoeae*** [a, b]
Susceptible	Isolates exhibiting *either* susceptibility *or* intermediate resistance to *both* penicillin and tetracycline	ß-lactamase negative isolate exhibiting: • Susceptibility to penicillin [MIC < 2.0 µg/ml (>26 mm)] • Susceptibility to tetracycline [MIC < 2.0 µg/ml (>30 mm)]
PPNG	Penicillinase-producing *Neisseria gonorrhoeae*	ß-lactamase positive isolate. Approximately six ß-lactamase plasmids have been identified in *N. gonorrhoeae,* most commonly: • "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 ß-lactamase and not by MICs of penicillin.) [c]
TRNG	Tetracycline resistant *Neisseria gonorrhoeae*	ß-lactamase negative isolates possessing a TetM-containing conjugative plasmid. TRNG isolates will exhibit both: • Susceptibility to penicillin [MIC < 2.0 µg/ml (>26 mm)] • Resistance to tetracycline with MIC ≥ 16.0 µg/ml (≤20 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 *Neisseria gonorrhoeae*	ß-lactamase positive isolates of *N. gonorrhoeae* exhibiting: • Resistance to tetracycline with MIC ≥ 16.0 µg/ml (<20 mm)
PenR	Chromosomally mediated resistance to penicillin	ß-lactamase negative isolates exhibiting both: • Resistance to penicillin with MIC ≥ 2.0 µg/ml (≤26 mm) • Susceptibility to tetracycline [MIC < 2.0 µg/ml (>30 mm)]
TetR	Chromosomally mediated resistance to tetracycline	ß-lactamase negative isolates exhibiting both: • 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 *Neisseria gonorrhoeae*	ß-lactamase negative isolates exhibiting both: • Resistance to penicillin with MIC ≥ 2.0 µg/ml (≤26 mm) • Resistance to tetracycline with MIC ≥ 2.0 µg/ml (≤30 mm)

[a] ***Note:*** Some TRNG may exhibit tetracycline MICs <16.0 µg/ml, and some TetR isolates may exhibit tetracycline MICs ≥16.0 µg/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 µg/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 (≥8.0 µg/ml) (≤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 µg/ml of penicillin but are still ß-lactamase positive.

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

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

Further characterization of resistant strains

An area of research in which reference laboratories may be interested in participating is the further subtype characterization[27] 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 ß-lactamase testing, providing the laboratory with phenotypic data appropriate for international comparisons.

Tests identifying gonococcal strains that produce ß-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, ß-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 ß-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 ß-lactamase production by* N. gonorrhoeae**

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

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

Results of ß-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 chocolatized 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: _____/_____/_____
*Test performed by:*_____

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

Specimen number	*Antimicrobial:* **Ciprofloxacin**	*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
NCCLS QC strain ATCC 49226 *QC in range?* →	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No
(Other QC strain)[a] *QC in range?* →	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.

Reveiwed 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 CO_2-supplemented atmosphere with increased humidity for 16–20 hours.
   - ***Note:*** if isolates are maintained in culture prior to inoculation for antimicrobial susceptibility testing, they must be subcultured every 24 hours prior to being tested.
   - ***Note:*** if isolates are stored frozen prior to inoculation for antimicrobial susceptibility testing, they must be subcultured at least once after initial culture from the frozen preparation prior to being tested.

c) Suspend isolated colonies (from the overnight cultures prepared in steps a and b) in 1.0–2.0 ml of Mueller-Hinton broth (or PBS). Mix the suspension thoroughly on a vortex mixer to break up clumps of growth as much as possible.
   - It is easier to prepare the suspensions with a swab[28] 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 51and 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 swab[28] in the standardized cell suspension and remove any excess moisture by rotating the swab against the glass above the liquid in the tube. Inoculate the entire surface of each plate three times, rotating the plate 60˚ each time to ensure confluent growth (Figure 34).

g) Store the inoculated plates at room temperature for 3–5 minutes to allow the medium to absorb the moisture from the inoculum. **It is essential that the surface of the medium is dry before the antimicrobial disks are applied.** Plates may be dried in an incubator or biohazard hood as described in step *e*. (If it takes longer than 15 minutes for the inoculum to dry, use a smaller volume / express more suspension from the swab in the future.)

h) Using sterile forceps, tweezers or a disk dispenser, apply disks of the selected antimicrobial agents to the surface of the inoculated medium; tap them to ensure they are in complete contact with the surface of the agar. **Once a disk has touched the agar surface, diffusion begins and it must not be moved.** All disks should be placed approximately the same distance from the edge of the plate and from each other (Figure 28).

i) Cover and invert the inoculated plates and incubate them at 35˚–36.5˚C in a 3%–5% CO_2 atmosphere (in a CO_2-incubator or candle-extinction jar) for 20–24 hours.

j) At 20–24 hours after inoculation and incubation, read the results of the antimicrobial susceptibility tests.

   - Examine the plates from the back, viewed from the top down against a black background and illuminated with reflected light (so hazy growth is more easily seen). Measure the diameter of the zone of inhibition with calipers, a ruler, or a ruler on a stick (see Figure 6).
   - Read the results for ATCC 49226 and compare them to the values in Table 9; if they are in control, continue reading and comparing results for the other

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

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

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

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

Antimicrobial susceptibility testing with the Etest® antimicrobial gradient strip[29] 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 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]

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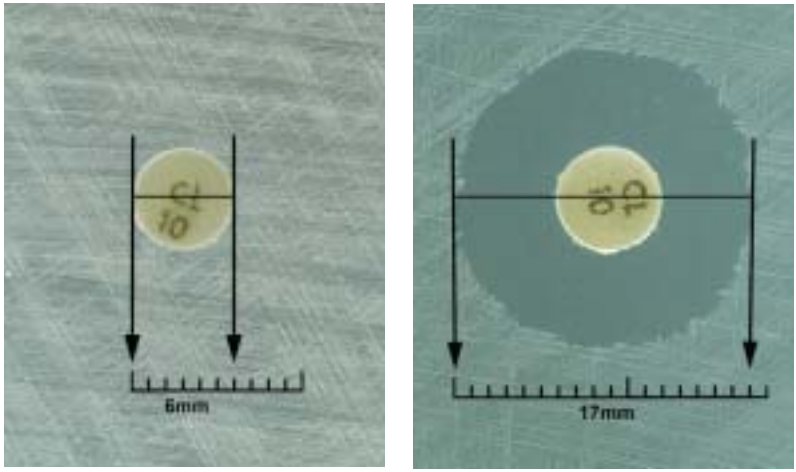
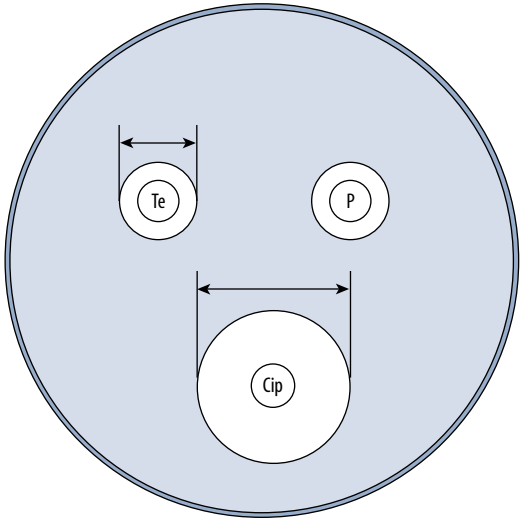
[29] The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor settings (see Appendix 13).

[30] Please note that for certain antimicrobial agents (particularly some ß-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 µg/ml – 32 µg/ml) rather than the high-range concentration (0.016 µg/ml – 256 µg/ml). A complete list of strips and ranges of concentrations is available from AB Biodisk (at http://www.abbiodisk.se/productsservice/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	P681E	CDC 10,328	CDC 10,329	SPJ-15	SPL-4
	Phenotype	***Susceptible***	***SpcR***	***PP-TR***	***CipI***	***CipR***	***"AznR"***	***"CfxDS"***
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> <*[ND]*>
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]*
ß-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; <[ND]> 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 ***N. gonorrhoeae*** 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 *(≤ 0.25µg/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% CO_2 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 *(CroDS)*	Isolates showing higher MICs or smaller zone sizes thanthese ranges should be retested to confirm the results, preferably with QC strains specifically for the antimicrobial agent showing abnormally high values. 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.
Cefixime	400 mg, single dose, oral	MIC > 0.25 µg/ml *(<31 mm)*	Decreased susceptibility *(CfxDS)*	
Ciprofloxacin	500 mg, single dose, oral	MIC 0.125 – 0.5 µg/ml *(28 - 40 mm)*	Intermediate resistance *(CipI)*	
		MIC ≥ 1.0 µg/ml *(≤ 27mm)*	Resistance *(CipR)*	
Ofloxacin	400 mg, single dose, oral	MIC 0.5 – 1.0 µg/ml *(25 – 30 mm)*	Intermediate resistance *(OfxI)*	
		MIC ≥ 2.0 µg/ml *(≤ 24 mm)*	Resistance *(OfxR)*	
Azithromycin	2 g, single dose, oral	MIC ≥ 1.0 µg/ml [b] *(≤ 25 mm)* [b]	'Resistance' [b] *('AznR')*	
Spectinomycin	2 g, single dose, intramuscular (IM)	MIC ≥ 128.0 µg/ml *(≤ 14 mm)*	Resistance *(SpcR)*	

[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.