Bacterial Agents of Enteric Diseases of Public Health Concern

Salmonella serotype Typhi
Shigella
Vibrio cholerae

Salmonella serotype Typhi

IDENTIFICATION AND
ANTIMICROBIAL SUSCEPTIBILITY TESTING

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

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

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

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

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

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

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

Identification of *S.* Typhi

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

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

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

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

Kligler iron agar and triple sugar iron agar

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

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

FIGURE 29: Flowchart for the isolation and identification of *Salmonella* ser. Typhi
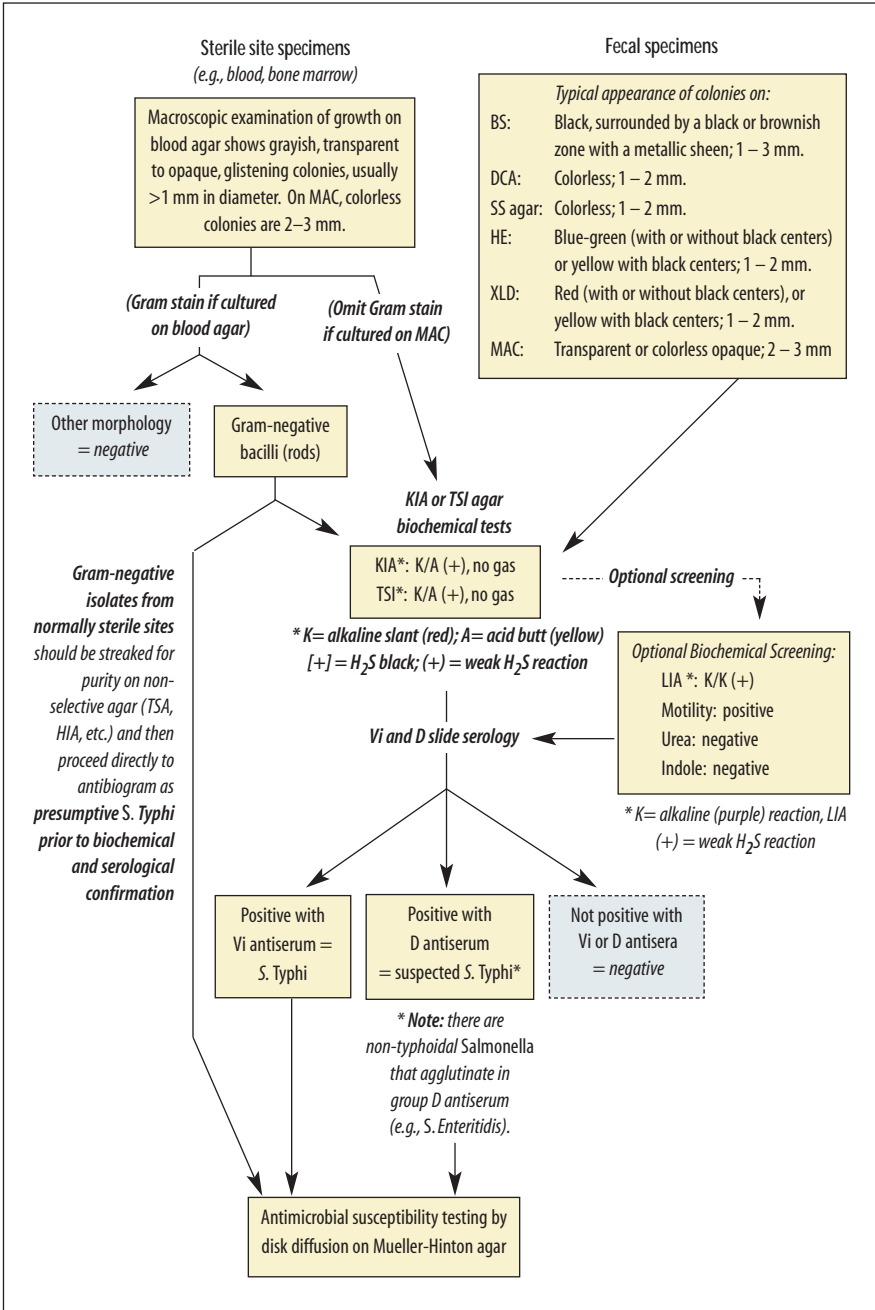
Sterile site specimens
(e.g., blood, bone marrow)

Macroscopic examination of growth on blood agar shows grayish, transparent to opaque, glistening colonies, usually >1 mm in diameter. On MAC, colorless colonies are 2–3 mm.

(Gram stain if cultured on blood agar)

(Omit Gram stain if cultured on MAC)

Other morphology = *negative*

Gram-negative bacilli (rods)

Gram-negative isolates from normally sterile sites should be streaked for purity on non-selective agar (TSA, HIA, etc.) and then proceed directly to antibiogram as **presumptive** S. **Typhi prior to biochemical and serological confirmation**

KIA or TSI agar biochemical tests

KIA*: K/A (+), no gas
TSI*: K/A (+), no gas

* K= alkaline slant (red); A= acid butt (yellow)
[+] = H$_2$S black; (+) = weak H$_2$S reaction

Vi and D slide serology

Positive with Vi antiserum = *S.* Typhi

Positive with D antiserum = suspected *S.* Typhi*

Not positive with Vi or D antisera = *negative*

** **Note:** there are non-typhoidal* Salmonella *that agglutinate in group D antiserum (e.g., S. Enteritidis).*

Antimicrobial susceptibility testing by disk diffusion on Mueller-Hinton agar

Fecal specimens

Typical appearance of colonies on:
BS: Black, surrounded by a black or brownish zone with a metallic sheen; 1 – 3 mm.
DCA: Colorless; 1 – 2 mm.
SS agar: Colorless; 1 – 2 mm.
HE: Blue-green (with or without black centers) or yellow with black centers; 1 – 2 mm.
XLD: Red (with or without black centers), or yellow with black centers; 1 – 2 mm.
MAC: Transparent or colorless opaque; 2 – 3 mm

- - - - *Optional screening* - - -

Optional Biochemical Screening:
LIA *: K/K (+)
Motility: positive
Urea: negative
Indole: negative

* K= alkaline (purple) reaction, LIA (+) = weak H$_2$S reaction

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

Specimen number	Agar medium [a]	Colony[b]	Gram stain[c]	KIA / TSI	LIA	Motility	Urea	Vi	D	Identification
						OPTIONAL		SLIDE SEROLOGY[d]		
		1								
		2								
		3								
		1								
		2								
		3								
		1								
		2								
		3								
		1								
		2								
		3								
		1								
		2								
		3								

a The choice of agar medium will depend on whether a specimen is from a normally sterile site (*e.g.*, blood, bone marrow, urine) or is a fecal specimen. Sterile site specimens should be cultured on blood agar; fecal specimens should be plated on selective media (such as BS, DCA, SS, HE, XLD, or MAC).
b If macroscopic examination of morphology reveals there is more than one type of colony on a plate, perform tests to identify each of the different isolates.
c Gram stain should only be performed on isolates from normally sterile sites (*e.g.*, blood, bone marrow, urine) and should not be performed on growth from MAC or other selective media.
d If prompt identification is required for clinical decision-making, slide serology may precede biochemical testing.

hours at 35°–37°C, the TSI or KIA slants are observed for reactions typical of *Salmonella.* **On TSI or KIA slants, *S.* Typhi characteristically produces an alkaline slant (red, "K"), an acid butt (yellow, "A"), and a small amount of blackening of the agar (H_2S, +) at the site of the stab on the slant and in the stab line (Figure 31); no gas (G) is produced.** It is worth noting that occasionally *S.* Typhi isolates do not produce H_2S. *S.* Paratyphi A isolates on TSI/KIA are usually K/AG and do not produce H_2S. Most other *Salmonella* serotypes produce a K/AG+ reaction, indicating that glucose is fermented with gas and H_2S production. Table 12 summarizes the reactions of *Salmonella* on screening biochemicals.

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

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

Lysine iron agar

Lysine iron agar (LIA) is a useful screening medium because most *Salmonella* isolates decarboxylate lysine and produce H_2S, whereas gas production varies by serotype. Preparation and quality control (QC) of this medium are described in Appendix 2. Inoculate LIA by stabbing the butt and streaking the surface of the slant; read and interpret the reactions after incubation for 24 hours at 35°–37°C for 24 hours.

On LIA, *Salmonella* typically give an alkaline (purple) reaction on the slant and butt and may produce gas and H_2S (blackening of medium) as well, as indicated in Table 12. When the reaction in the butt of the tube is alkaline, the lysine is decarboxylated and the isolate is termed "lysine-positive." Unlike most other *Salmonella, S.* Paratyphi A isolates are lysine-negative and appear yellow on LIA.

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

Motility agar

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

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

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

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

Screening medium	*Salmonella* Typhi	*Salmonella* Paratyphi A	Nontyphoidal *Salmonella* or *Salmonella* Paratyphi B or C
Triple sugar iron agar (TSI)	K/A(+)[a]	K/AG-[a]	K/AG+[a]
Kligler iron agar (KIA)	K/A(+)[a]	K/AG-[a]	K/AG+[a]
Lysine iron agar (LIA)	K/K(+)[b]	K/AG-[b]	K/K+[b]
Hydrogen sulfide (H$_2$S)	(weak)[c]	negative	positive
Urea	negative	negative	negative
Motility	positive[c]	positive	positive
Indole	negative	negative	negative

[a] for KIA / TSI: K = alkaline (red); A = acid (yellow); G = gas production; + = black H$_2$S produced (weak); − = no H$_2$S

[b] for LIA: K= alkaline (purple); A= acid (yellow); G = gas production; + = black H$_2$S produced (weak); − = no H$_2$S

~ An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated.

~ An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.

[c] this reaction occurs 97% of the time.

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

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

Urea medium

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

Slide serology for *S*. Typhi identification

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

will be weakly positive in both). Occasionally, *S.* Paratyphi C will also be positive in Vi antiserum, but because it produces gas from glucose and is H_2S positive, reactions on KIA/TSI allow for the differentiation of *S.* Paratyphi C from *S.* Typhi.

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

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

b) Emulsify the growth in three small drops of physiological saline and mix thoroughly.

c) Add a small drop of O group D antiserum to one of the suspensions and a small drop of Vi antiserum to a second. The third suspension is used as a control for autoagglutination (roughness). Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 ml can be used. A bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32).

d) Mix the suspension and antiserum thoroughly and then tilt the slide back and forth to observe for agglutination. It will be easier to see the agglutination if the slide is observed under a bright light and over a black background; if the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 42). Examine the saline suspension carefully to ensure that it is even and does not show clumping caused by autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped. Strong agglutination reactions are read as positive.

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

Antimicrobial susceptibility testing of *S.* Typhi

Treatment with an appropriate antimicrobial agent is crucial for patients with typhoid. Because recent reports have noted an increasing level of resistance to one or more antimicrobial agents in *S.* Typhi strains, isolates should undergo antimicrobial susceptibility testing as soon as possible. The disk diffusion method
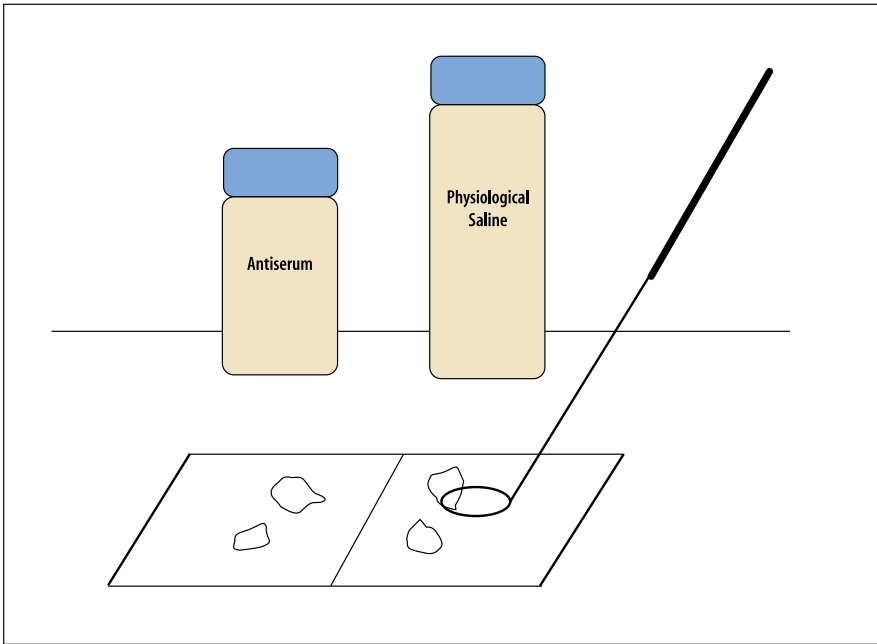
presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis),[31] and, if performed precisely according to the protocol below, will provide data that can reliably predict the *in vivo* effectiveness of the drug in question. However, any deviation from the method can invalidate the results. For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for antimicrobial susceptibility testing. Antimicrobial agents suggested for use in antimicrobial susceptibility testing of *S.* Typhi are listed in Table 13.

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

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

---

[31] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

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

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

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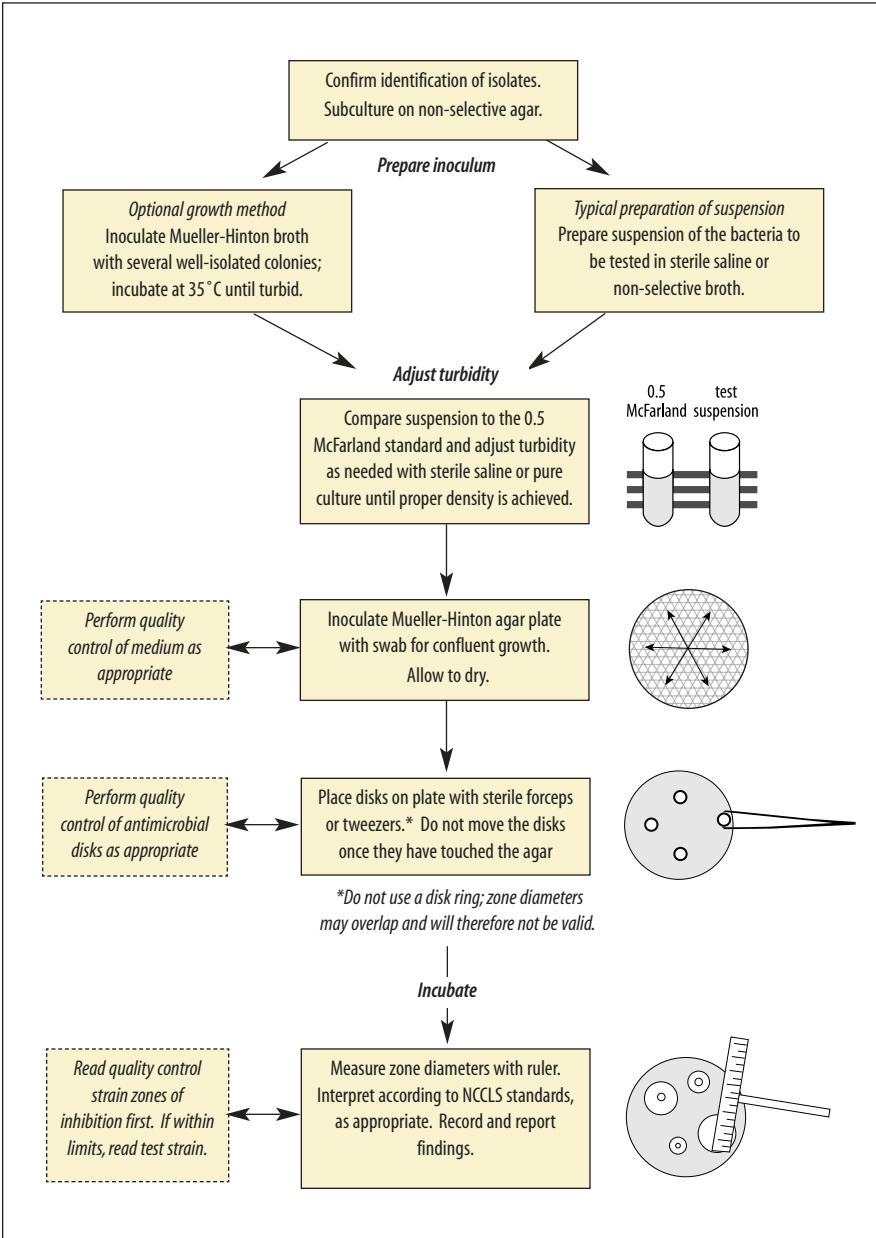
Agar disk diffusion testing of *S*. Typhi

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

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

Preparation of inoculum

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

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

Confirm identification of isolates.
Subculture on non-selective agar.

Prepare inoculum

Optional growth method
Inoculate Mueller-Hinton broth
with several well-isolated colonies;
incubate at 35°C until turbid.

Typical preparation of suspension
Prepare suspension of the bacteria to
be tested in sterile saline or
non-selective broth.

Adjust turbidity

Compare suspension to the 0.5
McFarland standard and adjust turbidity
as needed with sterile saline or pure
culture until proper density is achieved.

0.5   test
McFarland suspension

Inoculate Mueller-Hinton agar plate
with swab for confluent growth.
Allow to dry.

*Perform quality
control of medium as
appropriate*

Place disks on plate with sterile forceps
or tweezers.* Do not move the disks
once they have touched the agar

*Perform quality
control of antimicrobial
disks as appropriate*

*Do not use a disk ring; zone diameters
may overlap and will therefore not be valid.*

Incubate

Measure zone diameters with ruler.
Interpret according to NCCLS standards,
as appropriate. Record and report
findings.

*Read quality control
strain zones of
inhibition first. If within
limits, read test strain.*

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

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

Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 34). Finally, swab around the entire edge of the agar surface.
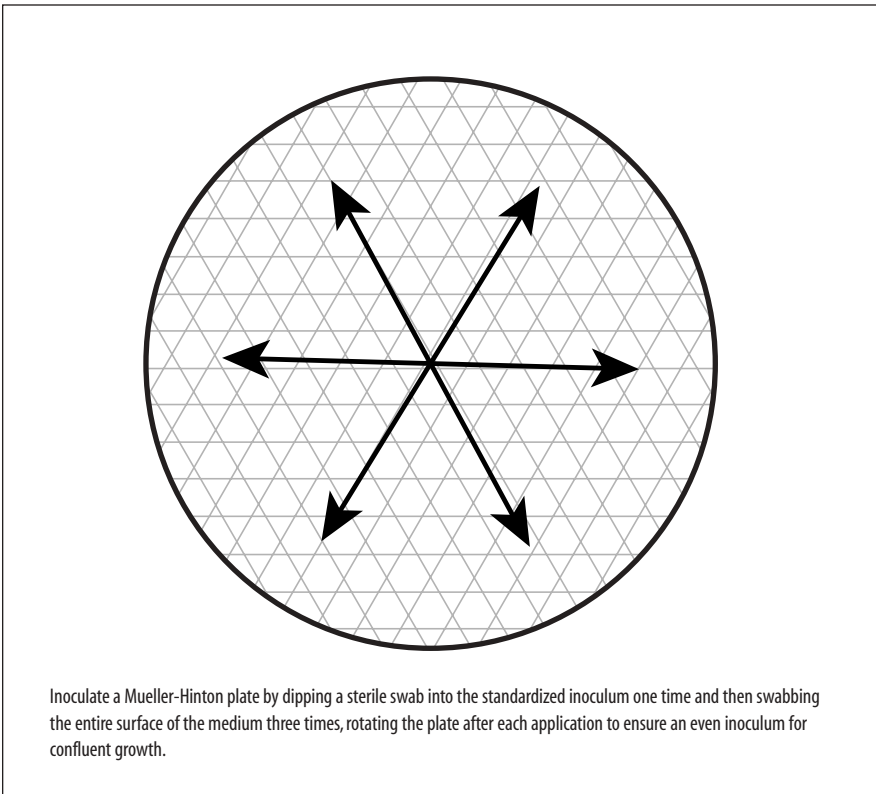
If the bacterial colonies used to prepare the suspension are picked off a plate containing mixed growth (*i.e.*, if isolated colonies are picked from a plate that does not contain pure culture, as may occur when working with cultures from stool specimens), laboratorians may choose to prepare a purity plate to ensure the suspension used for antimicrobial susceptibility testing is pure. To prepare the purity plate, after inoculating the Mueller-Hinton agar plate for confluent growth, label (a portion of) a separate TSA plate (or other non-selective medium) and **use the same swab** of suspension with which the Mueller-Hinton was inoculated to streak for isolation; do not place the swab back into the suspension. Several inocula can be streaked on different sections of a properly labeled purity plate, but the streaks **must not overlap.**

Quality control

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

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

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

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

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

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

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

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

Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

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

Antimicrobial disks

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

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

Recording and interpreting results

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

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

a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

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

Data for decision-making

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

• The antimicrobial agent should be affordable.

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

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

Date of Testing: ____/____/____

Test performed by: _____

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

Specimen number	Ampicillin	Chloramphenicol	Trimethoprim-sulfamethoxazole	Nalidixic acid [a]	(other drug)
	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm
	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm
	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm
	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm
	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm	S I R ___ mm
E. coli ATCC 25922 (NCCLS QC strain) QC in range? →	Yes No ___ mm	Yes No ___ mm	Yes No ___ mm	Yes No ___ mm	Yes No ___ mm

Reviewed by: _____   Date of Report: ____/____/____

[a] If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.

Note: After 16–18 hours incubation, check the results for the NCCLS-recommended quality control (QC) strain *E. coli* ATCC 25922 against the acceptable range of inhibition zone diameters and then record disk diffusion results (mm). (Inhibition zone ranges and breakpoints for interpretation of results may be found in Table 14.)

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

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

Shigella

IDENTIFICATION AND
ANTIMICROBIAL SUSCEPTIBILITY TESTING

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

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

• Only *S. dysenteriae* 1 causes large and prolonged epidemics of dysentery.

• Infection with *S. dysenteriae* 1 causes more severe, more prolonged, and more frequently fatal illness than does infection with other *Shigella*.

• Antimicrobial resistance develops more quickly and occurs more frequently in *S. dysenteriae* 1 than in other *Shigella* groups.

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

Identification of *Shigella*

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

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

Biochemical screening tests

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

Kligler iron agar and triple sugar iron agar

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

KIA and TSI tubes are inoculated by stabbing the butt and streaking the surface of the slant. After incubation for 18–24 hours at 35°–37°C, the slants are observed for reactions typical of *Shigella*. When incubating most biochemicals, caps should be loosened before placement in the incubator. This is particularly important when using KIA and TSI. **If the caps are too tight and anaerobic conditions exist in KIA or TSI, the characteristic reactions of *Shigella* may not occur and a misleading result could be exhibited.** In addition, the KIA and TSI tubes must be prepared so that the tubes have a deep butt (*i.e.*, approximately 3.5 cm) and a long slant (*i.e.*, approximately 2.5 cm). *Shigella* characteristically produces an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H_2S (see Table 15 and Figure 38). A few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI.

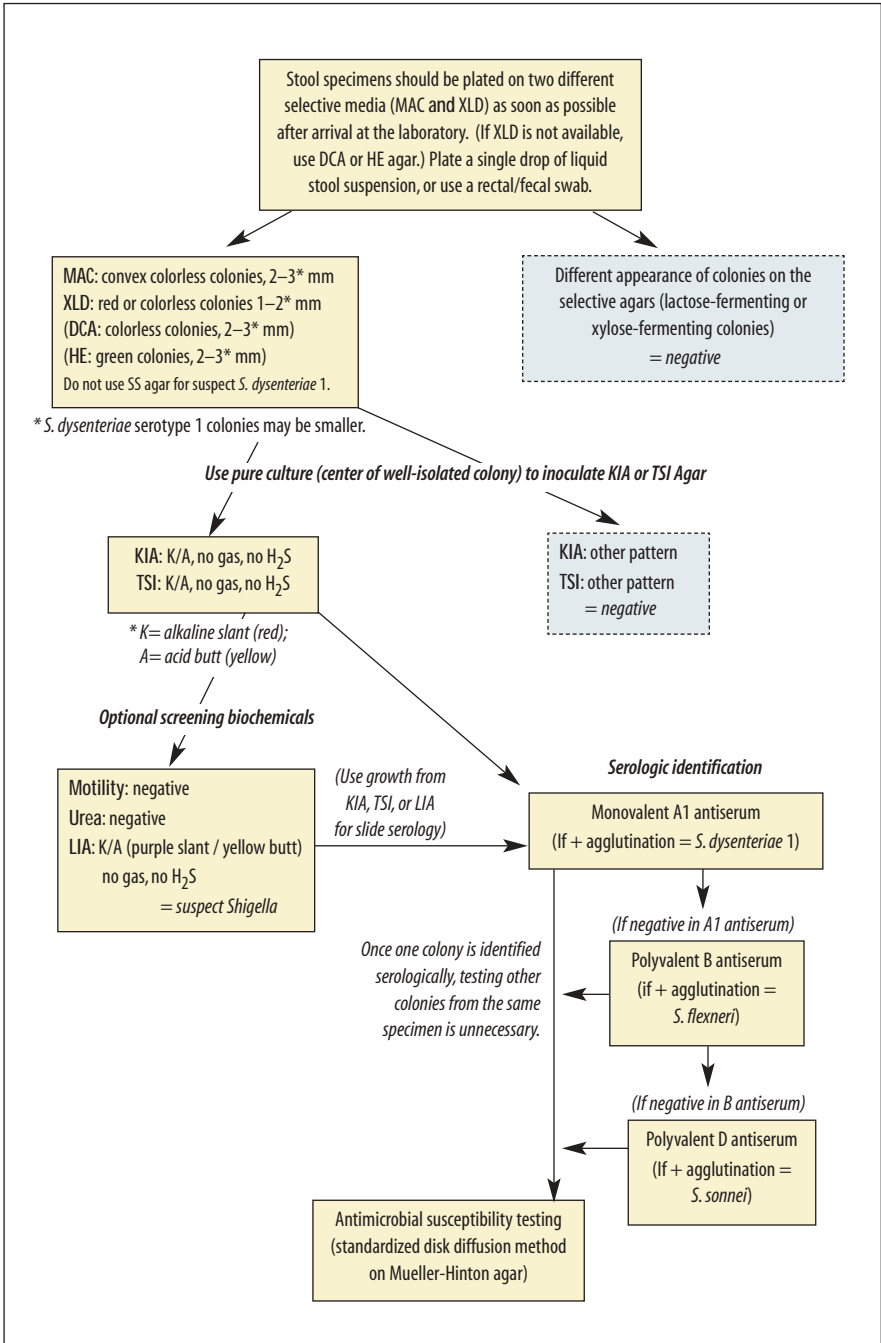FIGURE 36: Flowchart for the isolation and identification of *Shigella*

Stool specimens should be plated on two different selective media (MAC **and** XLD) as soon as possible after arrival at the laboratory. (If XLD is not available, use DCA or HE agar.) Plate a single drop of liquid stool suspension, or use a rectal/fecal swab.

MAC: convex colorless colonies, 2–3* mm
XLD: red or colorless colonies 1–2* mm
(DCA: colorless colonies, 2–3* mm)
(HE: green colonies, 2–3* mm)
Do not use SS agar for suspect *S. dysenteriae* 1.

Different appearance of colonies on the selective agars (lactose-fermenting or xylose-fermenting colonies)
= *negative*

* *S. dysenteriae* serotype 1 colonies may be smaller.

Use pure culture (center of well-isolated colony) to inoculate KIA or TSI Agar

KIA: K/A, no gas, no H_2S
TSI: K/A, no gas, no H_2S

KIA: other pattern
TSI: other pattern
= *negative*

* *K= alkaline slant (red);*
A= acid butt (yellow)

Optional screening biochemicals

Motility: negative
Urea: negative
LIA: K/A (purple slant / yellow butt)
no gas, no H_2S
= *suspect Shigella*

(Use growth from KIA, TSI, or LIA for slide serology)

Serologic identification

Monovalent A1 antiserum
(If + agglutination = *S. dysenteriae* 1)

(If negative in A1 antiserum)

Polyvalent B antiserum
(if + agglutination = *S. flexneri*)

Once one colony is identified serologically, testing other colonies from the same specimen is unnecessary.

(If negative in B antiserum)

Polyvalent D antiserum
(If + agglutination = *S. sonnei*)

Antimicrobial susceptibility testing
(standardized disk diffusion method on Mueller-Hinton agar)

FIGURE 37: Sample worksheet for *Shigella* test results

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

[a] XYL/LAC–: Xylose or Lactose -negative colonies
XYL/LAC+ : Xylose or Lactose -positive colonies
[b] Identification of only one colony from each suspect case must be confirmed as *Shigella*.

[c] A1 = Monovalent antiserum for *Shigella dysenteriae* (Serogroup A) serotype 1
B = Polyvalent antiserum for *Shigella flexneri* (Serogroup B)
D = Polyvalent antiserum for *Shigella sonnei* (Serogroup D)

TABLE 15: Reactions of *Shigella* in screening biochemicals

Screening medium	*Shigella* reaction	Figure number
Kligler iron agar (KIA)	K/A, no gas produced (red slant/yellow butt)[a]	*Figure 38*
Triple sugar iron agar (TSI)	K/A, no gas produced (red slant/yellow butt)[a]	~
H_2S (on KIA or TSI)	Negative *(positive reaction would be blackened medium)*	~
Motility	Negative	*Figure 39*
Urea	Negative	*Figure 40*
Indole	Positive or Negative	~
Lysine iron agar (LIA)	K/A (purple slant/yellow butt) [b]	*Figure 41*

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

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

Motility agar

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

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

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

Additional biochemical screening tests

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

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

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

FIGURE 40: Reactions in urea medium

Non-motile   Motile

A pink color develops in a positive urease reaction (*tube on left*)

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

Urea medium

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

Lysine iron agar

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

indicates H_2S production. Organisms lacking lysine decarboxylase produce an alkaline slant (purple) and an acid butt (yellow), no gas, and no H_2S. *Proteus* and *Providencia* species will often produce a red slant caused by deamination of the lysine. Lysine iron agar must be prepared so that the tubes have a deep butt (*i.e.*, approximately 3.5 cm). *Shigella* are lysine-negative and characteristically produce an alkaline (purple) slant, an acid (yellow) butt, no gas, and no H_2S in LIA.

Serologic identification of *Shigella*

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

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

Slide agglutination

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

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

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

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

TABLE 16: Subgroup and serotype designations of *Shigella*

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

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

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

[c] Because *S. boydii* is so rare it is not cost-effective to perform routine screens for it.

b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, heart-infusion agar (HIA), or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.

c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 µl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.

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

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

Antimicrobial susceptibility testing of *Shigella*

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

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

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

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

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

---

[32] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

- *Test the isolates from the beginning of an outbreak*
  Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. (World Health Organization [WHO] surveillance manuals can be useful guides for survey design.)

- *Test appropriate antimicrobial agents*
  The laboratory should routinely test only those antimicrobial agents that are available in the country or antimicrobial agents that are recommended by WHO as efficacious in the treatment of shigellosis (Table 17). In addition, if all isolates are resistant to a particular antimicrobial agent (*e.g.*, to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.

- *Provide timely feedback to public health officials*
  Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials. The data can then be used to make rational choices for antimicrobial treatment policy.

- *Monitor for changes in antimicrobial susceptibility*
  As a dysentery epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic. These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

Antimicrobial agents for treatment and testing of *Shigella*

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Inoculation procedure

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

5. Antimicrobial disks

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

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

6. Recording and interpreting results

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

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

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

a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

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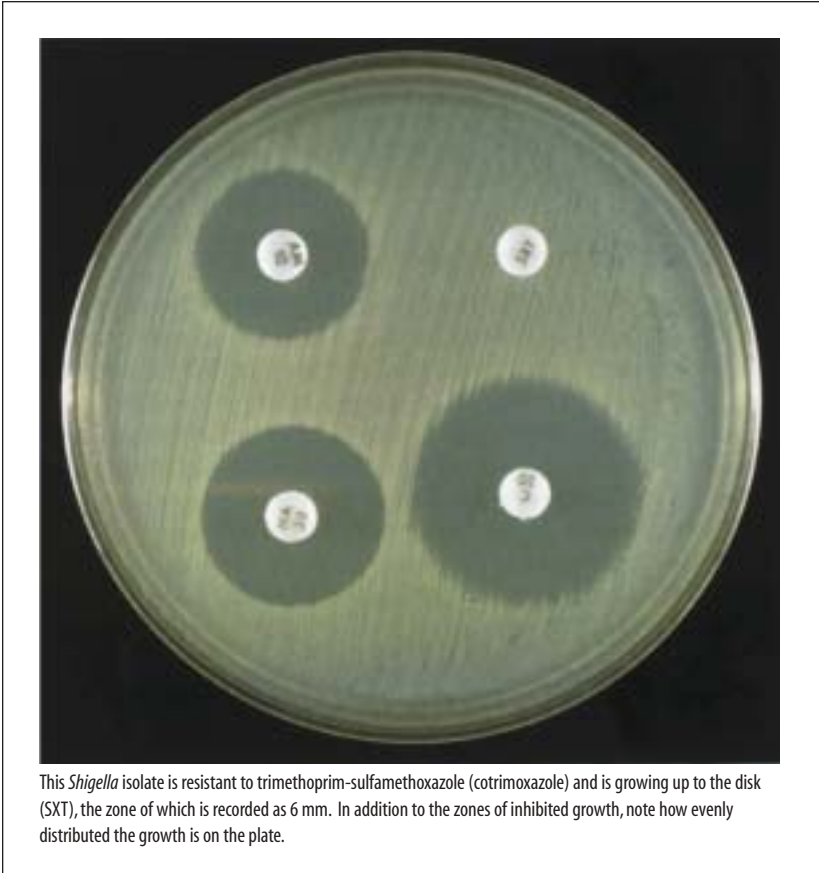
7. Quality control

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

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

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

Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

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

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

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

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

Date of Testing: ___ / ___ / ___

Test performed by: _____

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

Specimen number	Ampicillin		Chloramphenicol		Trimethoprim-sulfamethoxazole		Nalidixic acid [a]		(other drug) _____	
	S I R	mm	S I R	mm	S I R	mm	S I R	mm	S I R	mm
	S I R	mm	S I R	mm	S I R	mm	S I R	mm	S I R	mm
	S I R	mm	S I R	mm	S I R	mm	S I R	mm	S I R	mm
	S I R	mm	S I R	mm	S I R	mm	S I R	mm	S I R	mm
E. coli ATCC 25922 (NCCLS QC strain) **QC in range?** →	Yes No	mm	Yes No	mm	Yes No	mm	Yes No	mm	Yes No	mm

[a] If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin and will probably exhibit reduced susceptibility to ciprofloxacin.

Reviewed by: _____   ***Date of Report:*** ___ / ___ / ___

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

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

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

Data for decision-making: *informed epidemic response*

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

- The antimicrobial agent chosen should be effective against at least 80% of local *Shigella* strains.

- The antimicrobial agent chosen should be able to be given by mouth.

- The antimicrobial agent chosen should be affordable.

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

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

Vibrio cholerae
IDENTIFICATION AND
ANTIMICROBIAL SUSCEPTIBILITY TESTING

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

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

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

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

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

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

Identification of *V. cholerae*

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

Oxidase test

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

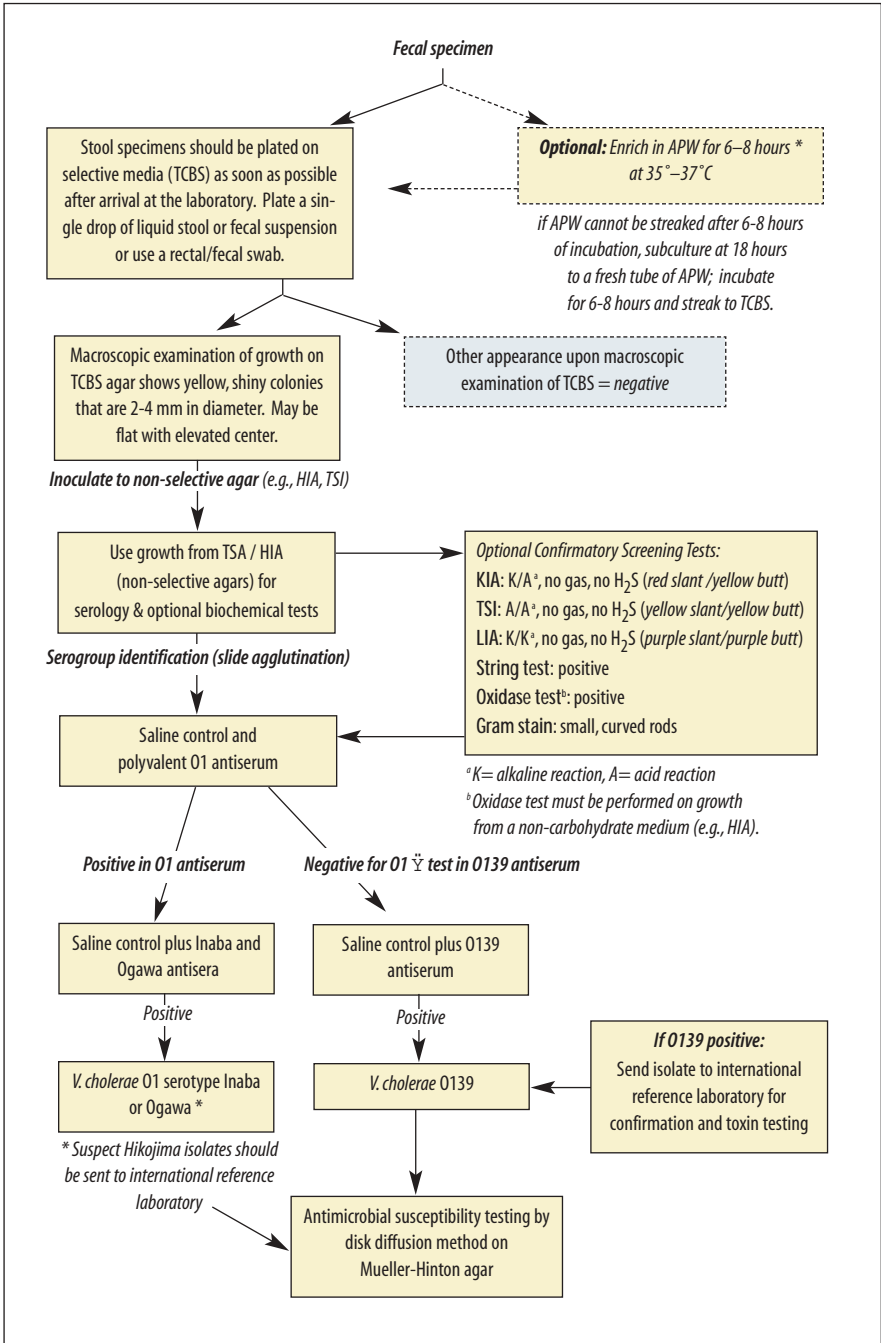FIGURE 45: Flowchart for isolation and identification of *Vibrio cholerae*

Fecal specimen

Stool specimens should be plated on selective media (TCBS) as soon as possible after arrival at the laboratory. Plate a single drop of liquid stool or fecal suspension or use a rectal/fecal swab.

Optional: Enrich in APW for 6–8 hours * at 35˚–37˚C

if APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours and streak to TCBS.

Macroscopic examination of growth on TCBS agar shows yellow, shiny colonies that are 2-4 mm in diameter. May be flat with elevated center.

Other appearance upon macroscopic examination of TCBS = *negative*

Inoculate to non-selective agar (e.g., HIA, TSI)

Use growth from TSA / HIA (non-selective agars) for serology & optional biochemical tests

Optional Confirmatory Screening Tests:
KIA: K/A[a], no gas, no H_2S (*red slant /yellow butt*)
TSI: A/A[a], no gas, no H_2S (*yellow slant/yellow butt*)
LIA: K/K[a], no gas, no H_2S (*purple slant/purple butt*)
String test: positive
Oxidase test[b]: positive
Gram stain: small, curved rods

[a] K= alkaline reaction, A= acid reaction
[b] Oxidase test must be performed on growth from a non-carbohydrate medium (e.g., HIA).

Serogroup identification (slide agglutination)

Saline control and polyvalent O1 antiserum

Positive in O1 antiserum

Negative for O1 ⅄ test in O139 antiserum

Saline control plus Inaba and Ogawa antisera

Saline control plus O139 antiserum

Positive

Positive

V. cholerae O1 serotype Inaba or Ogawa *

V. cholerae O139

If O139 positive:
Send isolate to international reference laboratory for confirmation and toxin testing

* Suspect Hikojima isolates should be sent to international reference laboratory

Antimicrobial susceptibility testing by disk diffusion method on Mueller-Hinton agar

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

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

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

[a] APW-TCBS : alkaline peptone water enrichment prior to inoculating TCBS
[b] SUC + : Sucrose-positive colonies
[c] SUC - : Sucrose-negative colonies

[d] PV01 = polyvalent antiserum for *V. cholerae* serogroup 01
Inaba= monovalent antiserum for *V. cholerae* 01 serotype Inaba
Ogawa= monovalent antiserum for *V. cholerae* 01 serotype Ogawa
0139 = monovalent antiserum for *V. cholerae* serogroup 0139

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

Moistened filter paper method

a) Add two to three drops of Kovac's oxidase reagent to a piece of filter paper in a petri dish and allow it to absorb; the filter paper should be moist (but not wet) after the reagent has been absorbed.

b) Using a platinum loop, a plastic loop, a sterile swab, or a sterile wooden applicator stick or toothpick, pick a portion of the colony to be tested **from non-selective media** and rub it onto the moistened filter paper. (**Do not use a Nichrome loop.**)

c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur in the region where the growth has been smeared within 10 seconds (Figure 10).

Swab method

a) Pick up suspect colonies from a non-selective culture plate or growth from a non-selective agar slant with the swab.

b) Use a Pasteur pipette to add one drop of Kovac's oxidase reagent to the swab.

c) If the isolate is *V. cholerae*, a positive (purple) reaction should occur within 10 seconds. (See Figure 20).

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

Additional biochemical screening tests

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

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

Screening test	*Vibrio cholerae* reactions	Figure number
Oxidase test	Positive	*Figure 10* and *Figure 20*
String test	Positive	*Figure 47*
Kligler iron agar (KIA)	K/A (red slant/yellow butt)[a], no gas produced, no H$_2$S [18–24 hours]	*Figure 48*
Triple sugar iron agar (TSI)	A/A (yellow slant/yellow butt)[a], no gas produced, no H$_2$S [18–24 hours]	*Figure 48*
Lysine iron agar (LIA)	K/K (purple slant / purple butt)[a,b], no gas produced, no H$_2$S [18–24 hours]	–
Gram stain	Small, gram-negative curved rods	–
Wet mount	Small, curved rods with darting motility	–

[a] K= alkaline; A= acid

[b] An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt indicates that lysine was not decarboxylated.

String test

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

Kligler iron agar and triple sugar iron agar

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

KIA or TSI agar slants are inoculated by stabbing the butt and streaking the surface of the medium. Incubate the slants at 35°–37°C and examine after 18–24 hours. **Caps on all tubes of biochemical media should be loosened before incubation,** but this is particularly important for KIA or TSI slants. **If the caps are too tight**
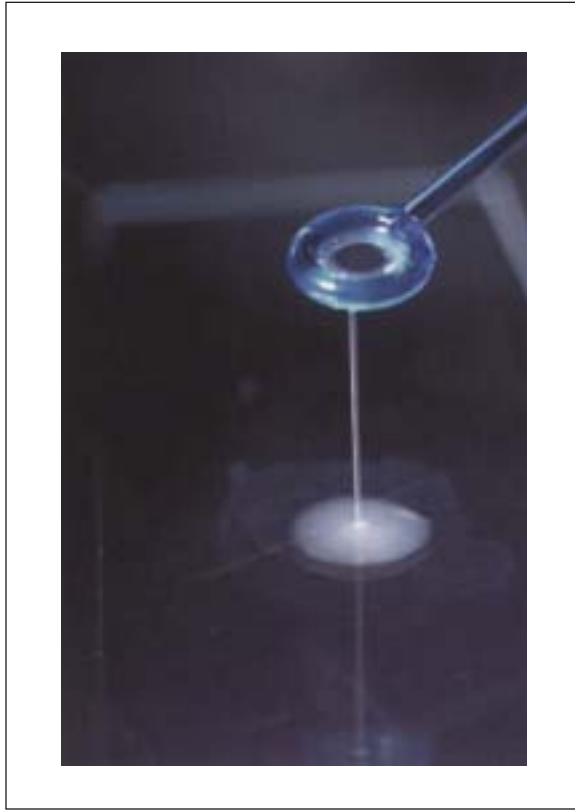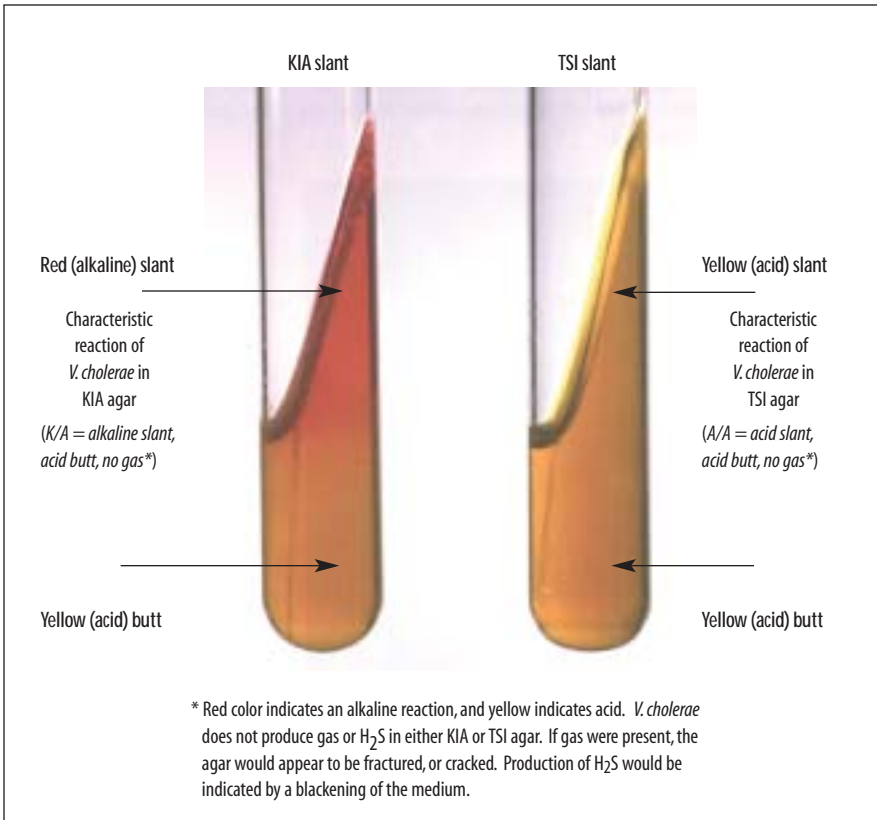
and anaerobic conditions exist in the tube, an inappropriate reaction will occur and the characteristic reactions of *V. cholerae* may not be exhibited.

The reactions of *V. cholerae* on KIA, which contains glucose and lactose, are similar to those of lactose-nonfermenting *Enterobacteriaceae* (*i.e.*, alkaline [red] slant, acid [yellow] butt, no gas, and no H_2S). However, on TSI, *V. cholerae* strains produce an acid (yellow) slant, acid (yellow) butt, no gas, and no H_2S (Table 19 and Figure 48).

Lysine iron agar

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

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

KIA slant   TSI slant

Red (alkaline) slant   Yellow (acid) slant

Characteristic
reaction of
V. cholerae in
KIA agar

(*K/A = alkaline slant,
acid butt, no gas**)

Characteristic
reaction of
V. cholerae in
TSI agar

(*A/A = acid slant,
acid butt, no gas**)

Yellow (acid) butt   Yellow (acid) butt

* Red color indicates an alkaline reaction, and yellow indicates acid. *V. cholerae*
   does not produce gas or H_2S in either KIA or TSI agar. If gas were present, the
   agar would appear to be fractured, or cracked. Production of H_2S would be
   indicated by a blackening of the medium.

streaking the slant; after incubation for 18–24 hours at 35°–37°C, examine the LIA
slants for reactions typical of *V. cholerae*. Organisms that produce lysine
decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the
tube (Figure 41); organisms without the enzyme produce an acid reaction (yellow
color) in the butt portion of the tube. H_2S production is indicated by a blackening
of the medium. The LIA reaction for *V. cholerae* is typically an alkaline slant
(purple), alkaline butt (purple), no gas, and no H_2S (Table 19). *Proteus* and
Providencia spp. will often produce a red slant caused by deamination of the lysine.

Gram stain

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

Wet mount

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

Serologic identification of *V. cholerae* O1 and O139

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

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

Presumptive identification using O1 and O139 antisera

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

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

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

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

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

Slide agglutination procedure

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

a) Divide the slide into test sections with a wax pencil and place one small drop of physiological saline in each test section on the slide.

b) Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, HIA, or other **non-selective** agar medium. (Serologic testing should not be done on growth from selective media such as MacConkey or XLD agar because selective media may yield false-negative serologic results.) Emulsify the growth in each drop of physiological saline on the slide and mix thoroughly to create a moderately milky suspension.

c) Add a small drop of antiserum to one of the suspensions; the second suspension serves as the control. To conserve antiserum, volumes as small as 10 μl can be used; a bent inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 32). Usually approximately equal volumes of antiserum and growth suspension are mixed, although the volume of suspension may be as much as double the volume of the antiserum.

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

V. cholerae O1 serotype	Ogawa antiserum	Inaba antiserum
Ogawa	$+^{a}$	$-^{b}$
Inaba	−	+
Hikojima [c]	+	+

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

^{b} − indicates a negative agglutination reaction in the absorbed antiserum.

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

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

Confirmation of V. cholerae *O139*

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

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

Antimicrobial susceptibility testing of *V. cholerae*

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

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

---

[33] Formerly known as the "National Committee for Clinical Laboratory Standards," NCCLS is now known solely by its acronym.

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

- *Test the isolates from the beginning of the outbreak*
  Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. That number will provide sufficient information to develop an antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns. WHO surveillance manuals can provide guidance in the development of such surveys.

- *Test appropriate antimicrobial agents*
  The laboratory should not routinely test antimicrobial agents that are not available in the country or antimicrobial agents that are not recommended by WHO as efficacious in the treatment of cholera (Table 21). In addition, if all isolates are resistant to a particular antimicrobial agent (*e.g.*, to ampicillin) during the first round of testing, testing against those agents during future surveys of the outbreak strain is probably not warranted (although testing of isolates may still be performed once or twice a year to confirm resistance). Sending 10 to 20 of the initial isolates to an international reference laboratory (Appendix 14) can be useful for confirmatory identification of the strain and antimicrobial susceptibility pattern. Guidelines for the packing and shipping of etiologic agents are included in Appendix 12.

- *Provide timely feedback to public health officials*
  Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials. The data can then be used to make rational choices for antimicrobial treatment policy.

- *Monitor for changes in antimicrobial susceptibility*
  As a cholera epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic. These surveys should be conducted every 2–6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials so that the antimicrobial treatment policy can be modified, if necessary. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation.

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

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

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

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

- Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, zone size interpretive criteria for *V. cholerae* O1 and O139 have been established by NCCLS only for ampicillin, chloramphenicol, sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole. The interpretations of susceptible, intermediate and resistant for isolates tested against these drugs by disk diffusion correlate well with the minimum inhibitory concentration (MIC) results determined by broth microdilution.

- Disk diffusion tests should not be used for doxycycline and erythromycin because the results for these drugs are frequently inaccurate for *V. cholerae* O1 and O139 strains. Therefore, these agents should not be tested using this method.

- The results from the tetracycline disk should be used to predict susceptibility to doxycycline. If a strain is susceptible to tetracycline, it will also be susceptible to doxycycline.

- At this time there is no *in vitro* method to accurately determine susceptibility to erythromycin.

- The reliability of disk diffusion results for other antimicrobial agents, including ciprofloxacin, furazolidone, and nalidixic acid, has not been validated.

  3 Until interpretive criteria have been established for *V. cholerae*, disk diffusion may be used to screen *V. cholerae* for resistance to ciprofloxacin, using NCCLS interpretive criteria for the *Enterobacteriaceae* (Table 22).

  3 Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* based on multi-laboratory studies using NCCLS testing methodologies. When screening with the disk diffusion method for these agents, results should be interpreted with caution (Table 22).

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

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

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

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

2. ***McFarland turbidity standard***

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

3. ***Preparation of inoculum***

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

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

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

4. *Inoculation procedure*

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

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

5. *Antimicrobial disks*

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

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

6. **Recording and interpreting results**

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

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

   a) If a purity plate was prepared, check the streak to confirm the culture was pure. (*Step a is optional.*)

   b) Record the diameter and interpretation of antimicrobial susceptibility of those colonies in the outer zone (*i.e.*, in addition to those in the inner zone).

   c) Pick the colonies inside the zone, streak for isolation on a new plate, confirm their identification, and perform the disk diffusion test again to confirm the previous results.

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

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

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

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

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

Date of Testing: _____ / _____ / _____

Test performed by: _____

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

Specimen number	Furazolidone [a]		Trimethoprim-sulfamethoxazole		Tetracycline [b]		Nalidixic acid [a]		*(other drug)* _____	
	mm		mm		mm		mm		mm	
	S *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*	
	mm		mm		mm		mm		mm	
	S *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*	
	mm		mm		mm		mm		mm	
	S *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*	
	mm		mm		mm		mm		mm	
	S *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*		*S* *I* *R*	
	mm		mm		mm		mm		mm	
E. coli ATCC 25922 (NCCLS QC strain) QC in range? →	**Yes** **No**		**Yes** **No**		**Yes** **No**		**Yes** **No**		**Yes** **No**	

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

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

Reviewed by : _____   Date: _____ / _____ / _____

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

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

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

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.

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

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

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

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

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

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

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

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

Data for decision-making: *informed epidemic response*

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

- The antimicrobial agent chosen should be effective against at least 80% of local *V. cholerae* O1/O139 strains. Evidence of clinical efficacy is the most important criterion, especially for a drug such as erythromycin, which cannot be tested *in vitro*.

- The antimicrobial agent chosen should be able to be given by mouth.

- The antimicrobial agent chosen should be affordable.

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

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

Conclusion

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

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

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

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