VI. LABORATORY IDENTIFICATION OF *VIBRIO CHOLERAE*

Members of the genus *Vibrio* are facultatively anaerobic, asporogenous, motile, curved or straight gram-negative rods. Vibrios either require NaCl or have their growth stimulated by its addition. All members of the genus *Vibrio*, with the exceptions of *V. metschnikovii* and *V. gazogenes*, are oxidase positive and reduce nitrates to nitrites. Within the *Vibrionaceae* are many different species, most of which are normal inhabitants of the aquatic environment. Of the more than 30 species within the *Vibrio-Photobacterium* complex, only 12 have been recognized as being pathogens for humans (Table V1-I). Although most of these 12 species are isolated from intestinal as well as extraintestinal infections, only *V. cholerae* is associated with epidemic cholera.

Unidentified vibrios have been called “marine species,” or simply, “marine vibrios.” These marine species are defined as *Vibrio* or *Photobacterium* strains that are oxidase positive, ferment D-glucose, do not grow in nutrient broth without added NaCl, but do grow in nutrient broth with add NaCl. Most organisms isolated from ocean or estuarian waters belong to the marine vibrio group and are difficult to identify except in a few specialized laboratories. Because they are not associated with human illness, marine vibrios need not be identified on a routine basis. Clinical and public health laboratories usually report the human pathogenic vibrios by genus and species and all other vibrios as “marine vibrio.”

The minimum identification of *V. cholerae* O1 requires only serologic confirmation of the presence of O1 serotype antigens with suspect isolates. However, a more complete characterization of the organism may be necessary and may include various biochemical tests as well as the determination of other characteristics. The laboratory should decide when it is appropriate to perform these additional tests on clinical isolates, since they should not be a routine part of identification of *V. cholerae* O1. Generally, if the isolate is from a region that is threatened by epidemic cholera or is in the early stages of a cholera outbreak, it is appropriate to confirm the production of cholera toxin and biochemical identification. Other tests that could provide important public health information include hemolysis, biotyping, molecular subtyping, and antimicrobial sensitivity assays. These tests should be performed on only a limited number of isolates. (See Chapter II, “The Role of the Public Health Laboratory.”)

A. Serologic Identification of *V. cholerae* O1

The use of antisera is one of the most rapid and specific methods of identifying *V. cholerae* O1. Although identifying the serogroup and serotype of *V. cholerae* isolates is not necessary for treatment of cholera, this information may be of epidemiologic and public health importance (Table VI-2.)

Table VI-1. Eight key differential tests to categorize the 12 clinically important *Vibrio* species into six groups (1)

Species	Growth in nutrient broth[a] 0% NaCl	Growth in nutrient broth[a] 1% NaCl	Oxidase	Nitrate to nitrate	Myo-inositol fermentation	Arginine dihydrolase	Lysine decarboxylase	Onithine decarboxylase
Group 1								
V. cholerae	+	+	+	+	-	-	+	+
V. mimicus	+	+	+	+	-	-	+	+
Group 2								
V. metschinikovii	-	+	-	-	V	V	V	-
Group 3								
V. cincinattiensis	-	+	+	+	+	-	V	-
Group 4								
V. hollisae	-	+	+	+	-	-	-	-
Group 5								
V. damsela	-	+	+	+	-	+	V	-
V. fluvialis	-	+	+	+	-	+	-	-
V. furnissii	-	+	+	+	-	+	-	-
Group 6								
V. alginolyticus	-	+	+	+	-	-	+	V
V.parahaeimolyticus	-	+	+	+	-	-	+	+
V. vulnificus	-	+	+	+	-	-	+	V
V. carchariae	-	+	+	+	-	-	+	-

Note: + = ≥90% positive; - = <10% positive; V = 10%-89% positive

[a] Difco Laboratories, Detroit, MI

Classification Method	Epidemic-associated	Not epidemic-associated
Serogroups	O1	Non-O1 (>130 exist)
Biotypes	Classical, El Tor	Biotypes not applicable to non-O1 strains
Serotypes	Inaba, Ogawa, Hikojima	These 3 serotypes not applicable to non-O1 strains
Toxin	Produce cholera toxin[a]	Usually do not produce cholera toxin; sometimes produces other toxins

Table VI-2. Characteristics of *Vibrio cholerae*

[a] Nontoxigenic O1 strains exist, but are not epidemic-associated

1. Serogroups of *V. cholerae*

Currently, there are more than 130 serogroups of *V. cholerae,* based on the presence of somatic O antigens. However, only the O1 serogroup is associated with epidemic and pandemic cholera. Other serogroups may be associated with severe diarrhea, but do not possess the epidemic potential of the O1 isolates and do not agglutinated in O1 antisera. Isolation of *V. cholerae* non-O1 from environmental sources in the absence of diarrheal cases is common. Laboratories may choose not to report the isolation of *V. cholerae* non-O1 when investigating cholera epidemics, since health care providers or public health officials may be unaware of the important epidemiologic differences between O1 and non-O1 isolates. The name "*Vibrio cholerae*" on a laboratory report may incorrectly imply that a non-O1 isolate is of epidemiologic importance. Confusion may be eliminated by reporting only whether *V. cholerae* serogroup O1 was or was not isolated.

2. Serotypes of *V. cholerae* O1

Isolates of the O1 serogroup of *V. cholerae* have been further divided into three serotypes, Inaba, Ogawa, and Hikojima (very rare). Serotype identification is based on agglutination in antisera to type-specific O antigens (see Table VI-3). Identifying these antigens is valid only with serogroup 1 isolates. For this reason, Inaba and Ogawa antisera should never be used with strains that are negative with polyvalent O1 antisera. Isolates that agglutinate weakly or slowly

with serogroup O1 antisera but do not agglutinate with either Inaba or Ogawa antisera are not considered to be serogroup O1.

Table VI-3. Identifying characteristics of serotypes of *V. cholerae* serogroup O1

Serotype	Major O factors Present	Agglutination in absorbed serum	
		Ogawa	Inaba
Ogawa	A, B	+	-
Inaba	A, C	-	+
Hikojima	A, B, C	+	+

Strains of one serotype frequently cross-react slowly and weakly in antiserum to the other serotype, depending on how well the serotype-specific antisera have been absorbed. Agglutination reactions with both 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.”

3. Slide agglutination

Agglutination tests for *V. cholerae* somatic O antigens may be carried out in a petri dish or on a clean glass slide. An inoculating needle or loop, or sterile applicator stick, or tooth pick is used to remove a portion of the growth from the surface of heart infusion agar (HIA), Kligler’s iron agar (KIA), triple sugar iron agar (TSI), or other nonselective agar medium. Emulsify the growth in a small drop of physiological saline and mix thoroughly by tilting back and forth for about 30 seconds. Examine the suspension carefully to ensure that it is even and does not show clumping due to autoagglutination. If clumping occurs, the culture is termed “rough” and cannot be serotyped.

If the suspension is smooth (turbid and free-flowing), add a small drop of antiserum to the suspension. 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 10µl can be used.

Mix the suspension and antiserum well and then tilt slide back and forth to observe for agglutination. If the reaction is positive, very strong clumping will appear within 30 seconds to 1 minute (Figure VI-1).

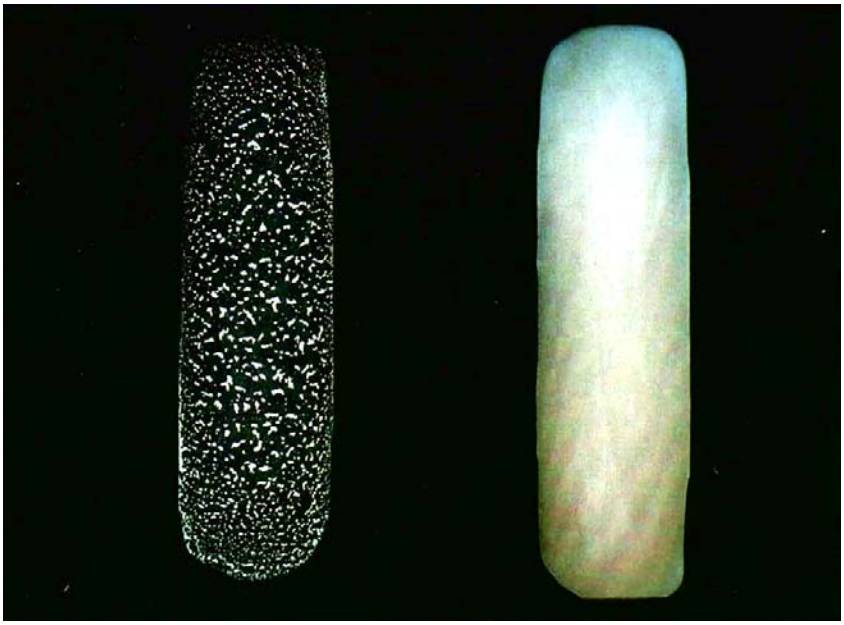

Figure V1-1. Antisera to the O1 serogroup of *V. cholerae* will agglutinate homologous organisms (left). A normal serum or saline control (right) does not show agglutination

Test	% positive

Table VI-4. Biochemical characteristics of typical isolates of *V. cholerae* O1

Oxidase	100
String test	100
Kligler's iron agar	K/A, no gas, no H_2S
Triple sugar iron agar	A/A, no gas, no H_2S
Glucose[a] (acid production)	100
Glucose (gas production)	0
Sucrose (acid production)	100
Lysine[a]	99
Arginine[a]	0
Ornithine[a]	99
Growth in O% NaCl[b]	100
Growth in 1% NaCl[b]	100
Voges-Proskauer[a]	75[c]

[a] Modified by the addition of 1% NaCl.
[b] Nutrient broth base (Difco Laboratories)
[c] Most isolates of *V. cholerae* serotype O1 biotype El Tor are positive in the VP test, whereas biotype classical strains are negative.

B. Biochemical Identification of *V. cholerae*

Since confirmation of *V. cholerae* O1 requires only identification of the O1 serotype antigens by slide agglutination, biochemical confirmation is only infrequently necessary (see Chapter II, "The Role of the Public Health Laboratory.") The tests listed in Table VI-4 constitute a short series of biochemicals that may be used to confirm isolates of *V. cholerae.* If the results of the tests with an isolate are the same as those shown in Table VI-4, the identity of the isolate is confirmed as *V. cholerae*. However, if the isolate does not give results as shown in the table, additional tests will be necessary for identification. See Chapter XI, "Preparation of Media and Reagents," for instructions for preparing media and reagents for the biochemical tests shown in Table VI-4. The use of KIA or TSI, the oxidase and "string" tests, and arginine or lysine reactions may be helpful for screening isolates resembling *V. cholerae*. Screening procedures for fecal and environmental specimens are discussed in Chapters IV and V.

1. Oxidase test

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate-containing medium. Do not use growth from thiosulfate citrate bile salts sucrose (TCBS) agar. Place 2 to 3 drops of oxidase reagent (1% tetramethyl-*p*-phenylenediamine) on a piece of filter paper in a petri dish. Smear the culture across the wet paper with a platinum (not nichrome) loop, a sterile wooden applicator stick, or toothpick. In a positive reaction, the bacterial growth becomes dark purple within 10 seconds (Figure VI-2). Color development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio, Neisseria, Campylobacter, Aeromonas, Plesiomonas*,

Pseudomonas, and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.

2. String test

The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from HIA 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 VI-3). Most vibrios are positive, whereas *Aeromonas* strains are usually negative.

3. Kligler's iron agar or triple sugar iron agar

KIA and TSI are carbohydrate-containing screening media widely used in diagnostic microbiology. While similar in use, the two media vary in the carbohydrates they contain. The reaction of *V. cholerae* on KIA, which contains glucose and lactose, is similar to those of non-lactose-fermenting *Enterobacteriaceae* (K/A, no gas, no H_2S)

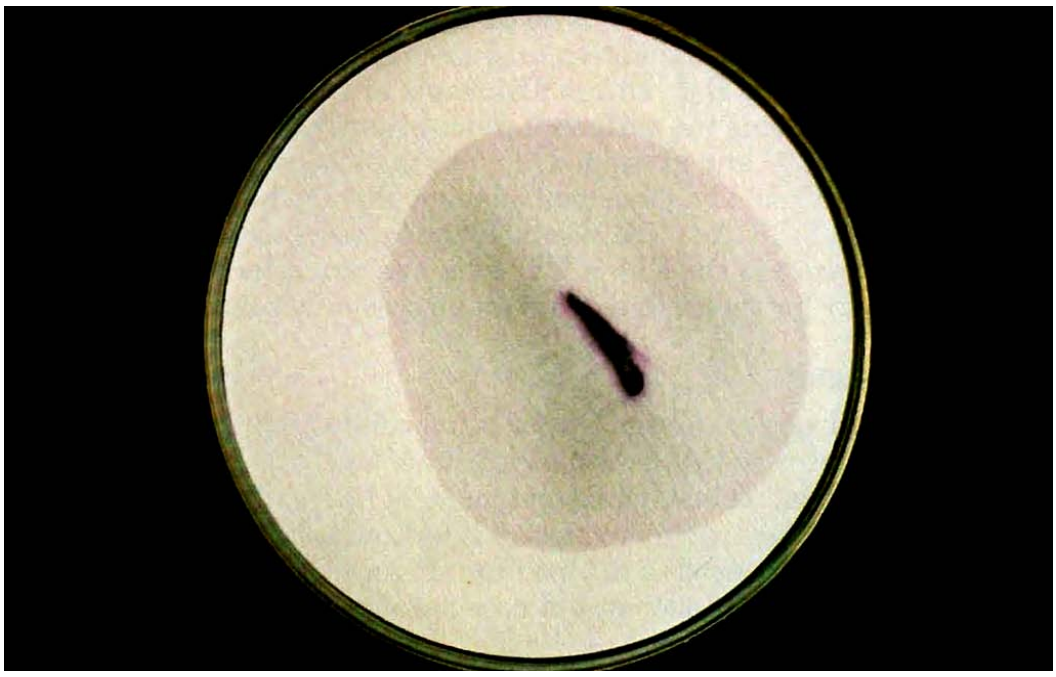

Figure VI-2. A positive oxidase test (as shown here) results in the development of a dark purple color within 10 seconds. *V. cholerae* is oxidase-positive, which differentiates it from oxidase-negative organisms such as the *Enterobacteriaceae*.

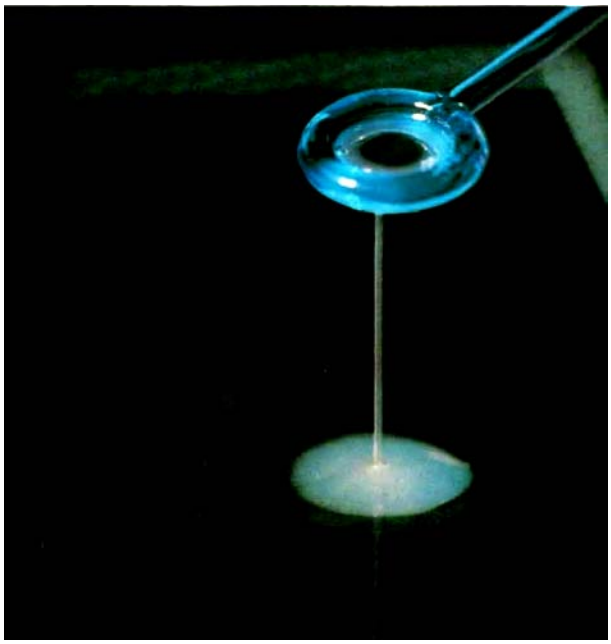

Figure VI-3. A positive string test, shown here with *V. cholerae*, is a rapid and simple method for distinguishing between the genus *Vibrio* (mostly positive) and *Aeromonas* (nearly always negative).

(Figure VI-4). TSI which contains sucrose in addition to glucose and lactose gives reactions of A/A, no gas, and no H_2S. KIA or TSA slants are inoculated by stabbing the butt and streaking the surface of the medium. Slants should be incubated at 35˚ to 37˚C and examined after 18 to 24 hours. Caps on all tubes of biochemicals 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 KIA or TSI tube, the characteristic reactions of *V. cholerae* may not be exhibited an inappropriate reaction will occur.

4. Carbohydrates

Glucose and sucrose broths should be inoculated lightly from fresh growth. The broths should be incubated at 35˚ to 37˚C and read at 24 hours. If fermentation tests are negative at 24 hours, they should be incubated for up to 7 days. Acid production is indicated by a pink color when Andrade indicator is used in the medium (Figure VI-5). *V. cholerae* ferments both glucose and sucrose but does not produce gas in either carbohydrate.

5. Decarboxylase/dihydrolase reactions

Arginine, lysine, ornithine, and control (without amino acid) broths modified by the addition of 1% NaCl should be inoculated lightly from a fresh culture. The broth in each tube should be overlayed with 4 to 5 mm of sterile mineral oil. Incubate at 35˚ to 37˚C and read at 24 to 48 hours, but if the test is negative it should be incubated for up to 7 days. When bromcresol purple and cresol red are used as indicators, an alkaline (positive) reaction is purple, while a negative or acid reaction is indicated by a yellow color (Figure VI-6). The test is valid only if the control tube stays negative (yellow). *V. cholerae* is typically positive for lysine decarboxylase, while certain other *Vibrio* spp. are negative and *Aeromonas* spp. are variable. *V. cholerae* is typically negative for arginine dihydrolase, while *Aeromonas*, *Plesiomonas*, and certain other *Vibrio* spp. are positive. *V. cholerae* is positive for ornithine decarboxylase.

Lysine iron agar and arginine glucose slant

A lysine iron agar (LIA) slant may be used instead of lysine broth (above) to test for the production of lysine decarboxylase; similarly, an arginine glucose slant (AGS; U.S. Food and Drug Administration. Bacteriological Analytical Manual, 6th ed. Arlington, Virginia: Association of Official Analytical Chemists, 1992) may be used instead of arginine broth to test for the production of arginine dihydrolase. These slants are used most frequently as part of a screening procedure (see Chapters IV and V). LIA and AGS should be inoculated by stabbing the butt and streaking the slant. Organisms that produce lysine decarboxylase in LIA (or arginine dihydrolase In AGS) cause an alkaline reaction (purple color) throughout the medium. Organisms without these enzymes typically produce an alkaline slant (purple) and an acid butt (yellow). *V. cholerae* give a K/K reaction in LIA (lysine positive) but produces a K/A reaction (arginine negative) in AGS.

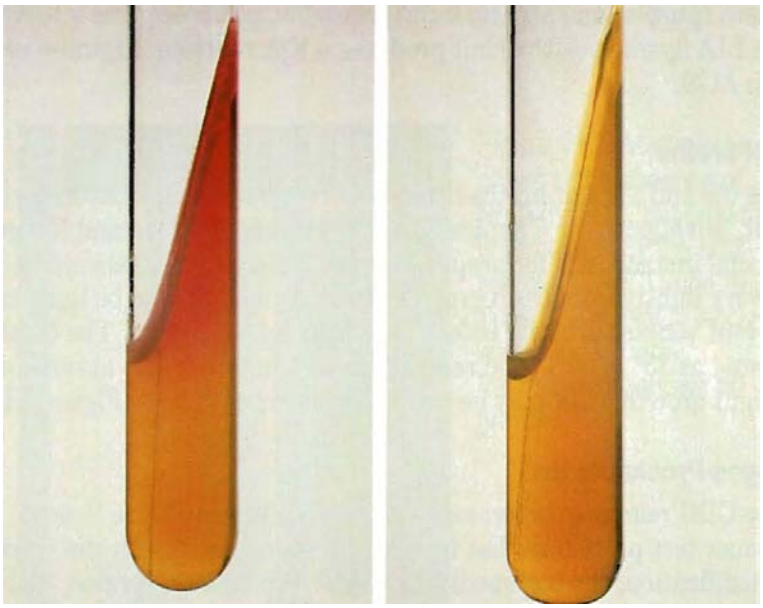

Figure VI-4. Reactions of *V. cholerae* in Kligler's iron agar (left) and triple sugar iron agar (right)

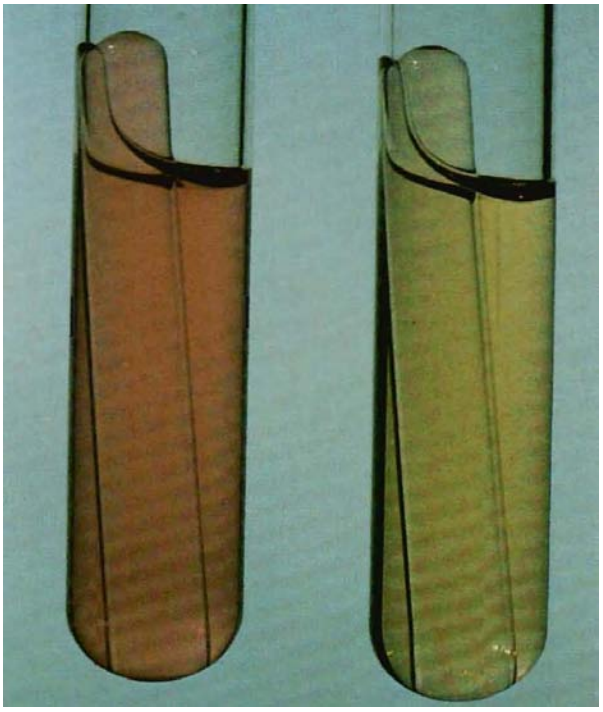

Figure VI-5. With Andrade indicator in the carbohydrate medium, a pink color develops if fermentation has occurred, while negative reactions appear yellow.

6. Salt broths

The 0% and 1% salt broths (nutrient broth base [Difco Laboratories, Detroit, Michigan]; see Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of salt test broths) should be inoculated very lightly from fresh growth. The inoculum should be light enough to prevent visible turbidity before incubation of the broths. The broths are incubated at 35˚ to 37˚C and read at 18 to 24 hours. In the absence of overnight growth, they may be incubated for up to 7 days (Figure VI-7).

7. Voges-Proskauer test

The CDC reference laboratory uses a modification of the Voges-Proskauer test procedure that increases its sensitivity with the vibrios. In this modification, the test medium (MR-VP broth) incorporates 1% NaCl, reagent A consists of 5% alpha-naphthol in absolute ethanol, and reagent B is a solution of 0.3% creatine in 40% KOH (potassium hydroxide). The test organism is incubated in MR-VP broth for 48 hours before reagents A and B are added. A cherry red color indicates a positive reaction (Figure VI-8).

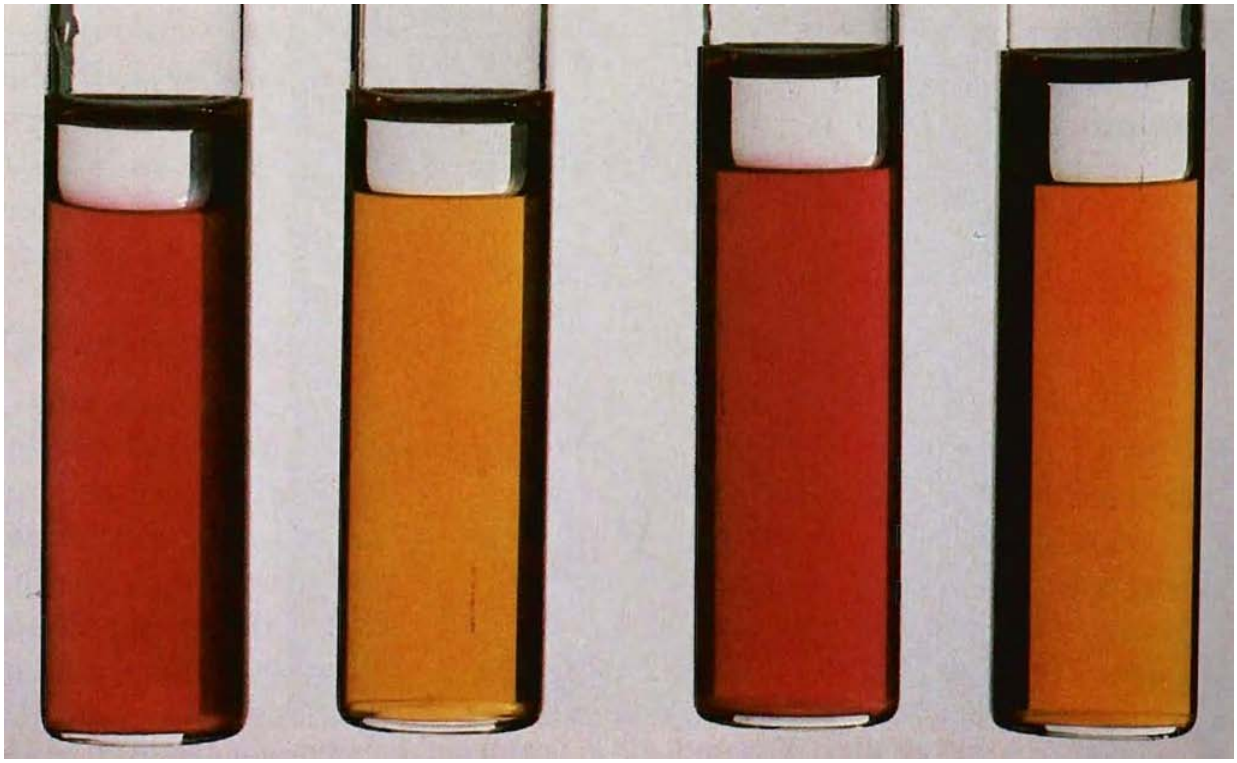

Figure VI-6. Decarboxylase and dihydrolase reactions for *V. cholerae* are, from left to right, lysine (+), arginine (-), ornithine (+), and control (-).

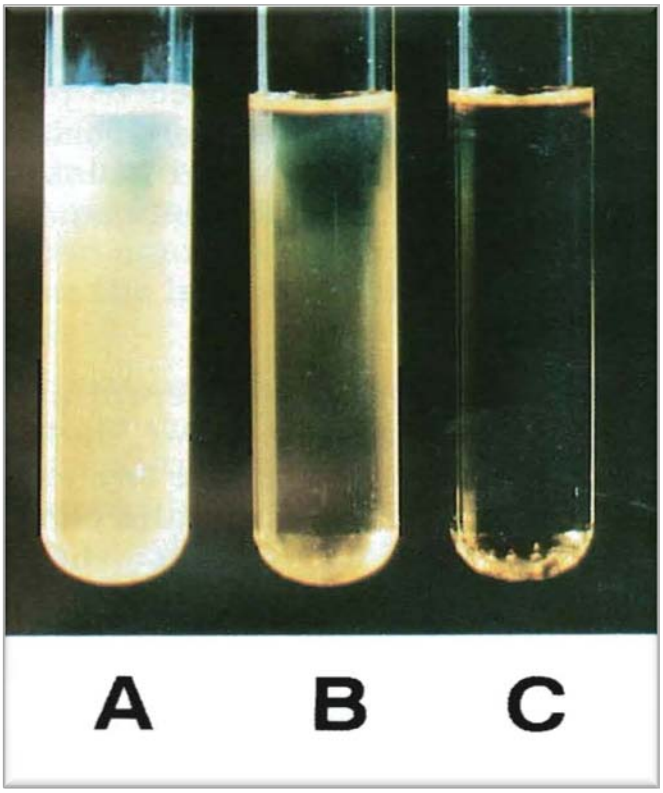

Figure VI-7. *V. cholerae* grows in the absence of NaCl (tube B), but growth is stimulated by the addiction of 1% NaCl (tube A). Tube C, 0% NaCl, inoculated with *V. parahaemolyticus*, shows no growth.

Figure VI-8. *V. cholerae* produces acetoin, which is detected in the Voges-Proskauer test, giving a red (positive) reaction (left). A negative reaction is on the right.

8. Susceptibility to vibriostatic compound O/129

Susceptibility to 2,4-diamino-6,7-diisopropyl-pteridine phosphate (referred to as O/129 or vibriostatic compound) has been recommended and used as a primary means for differentiating between *Vibrio* (which are sensitive to O/129) and *Aeromonas* (resistant to O/129). While most isolates of *V. cholerae* have been sensitive to O/129, several recent reports have described clinical and environmental isolates that were resistant to this compound. In these reports *V. cholerae* O1 and non-O1 isolates were resistant to 10 and 150µ of O/129, thus resembling *Aeromonas*. Caution should be exercised when relying on this test.

C. Hemolysis Testing

Historically, the classical and El Tor biotypes were differentiated by the ability of the El Tor group to lyse erythrocytes. However, by 1972 almost all El Tor isolates worldwide were nonhemolytic. The two exceptions to this trend have been the U.S. Gulf Coast and the Australia clones of *V. cholerae* O1, which are strongly hemolytic when assayed by either the plate or tube hemolysis assay (Table VI-5). For this reason, hemolysis continues to be a useful phenotypic characteristic for differentiating the Gulf Coast and Australia clones of *V cholerae* O1 from El Tor strains from the rest of the world, including Latin America.

1. Plate hemolysis

Blood agar plates containing 5% to 10% sheep blood should be streaked to obtain isolated colonies. Plates should be incubated at 35˚ to 37˚C for 18 to 24 hours. Hemolytic colonies should have clear zones around them where red blood cells have been totally lysed, and a suspected hemolytic strain should be compared with a strongly hemolytic control strain (Figure VI-9). Strains that give incomplete hemolysis (incomplete clearing of the red blood cells) should not be reported as hemolytic.

Table VI-5. Hemolytic activity of *V. cholera* O1 classical and El Tor biotypes

Biotype/location	Hemolytic activity
Classical	Negative
El Tor/Australia	Strongly positive
El Tor/US Gulf Coast	Strongly positive
El Tor/Latin America	Negative
El Tor/Asia, Africa, Europe, Pacific[a]	Negative

[a] Strains isolated between 1963 and 1992

On aerobic sheep blood agar plates, nonhemolytic *V. cholerae* frequently produces greenish clearing around areas of heavy growth but not around well-isolated colonies. This phenomenon, often describes as “hemodigestion,” is produced by metabolic by-products which are inhibited by anaerobic incubation of the blood agar plate. For this reason, when aerobic growth conditions are used, hemolysis should be determined around isolated colonies, not in areas of confluent growth. Also, aerobic blood agar plates should be incubated for no more than 18 to 24 hours, since the hemodigestion effect is accentuated during longer incubation periods.

Aerobic incubation of the plate for no longer than 24 hours, although not optimal for detection of hemolysis, will permit differentiation of strongly hemolytic strains, such as the U.S. Gulf Coast and Australia clones, from the nonhemolytic Latin American strains. If the results of the plate hemolysis assay are not conclusive, test the strain by the tube hemolysis method, which is less susceptible to misinterpretation than the plate method.

2. Tube hemolysis assay

Controls: Use two well-characterized strains of *V. cholerae*. One should be strongly hemolytic, the other nonhemolytic.

1) Wash 20 ml of sheep erythrocytes in 25 ml of phosphate-buffered saline (PBS), 0.01 M, pH 6.8-7.2. Repeat twice for a total of 3 washes. Prepare a 1% (vol/vol) suspension of packed sheep erythrocytes in PBS.
2) From fresh growth, inoculate test strains and controls into heart infusion broth (or Trypticase soy broth) with 1% glycerol (pH 7.4) and incubate at 35˚ to 37˚C for 24 hours. After incubation, centrifuge to sediment bacterial cells; remove the supernatants with a Pasteur pipette.
3) Divided the supernatants into two equal portions. One aliquot is heated to 56˚C for 30 minutes. Make serial twofold dilutions of both the heated and unheated supernatants in PBS (dilute to 1:1,024).
4) Add 0.5 ml of the 1% suspension of sheep erythrocytes in PBS to 0.5 ml of each dilution of supernatant.
5) Incubate in a water bath at 37˚C for 2 hours. Remove the suspensions from the water bath, and hold overnight at 4˚C.
6) Examine for evidence of hemolysis. Nonhemolyzed red blood cells will settle to the bottom of the test tube and form a “button” (Figure VI-10). No button will be present if the cells are lysed by hemolysin. Hemolysin titers should be recorded as the highest dilution at which complete hemolysis has occurred.
7) Compare results of the heated and unheated supernatants. Heated tubes should show no hemolysis, since the hemolysin of *V. cholerae* is heat-labile, and if present, is inactivated by the 56˚C incubation (step 3, above). Titers of 2 to 8 are considered intermediate, and titers of 16 or above are strongly positive.

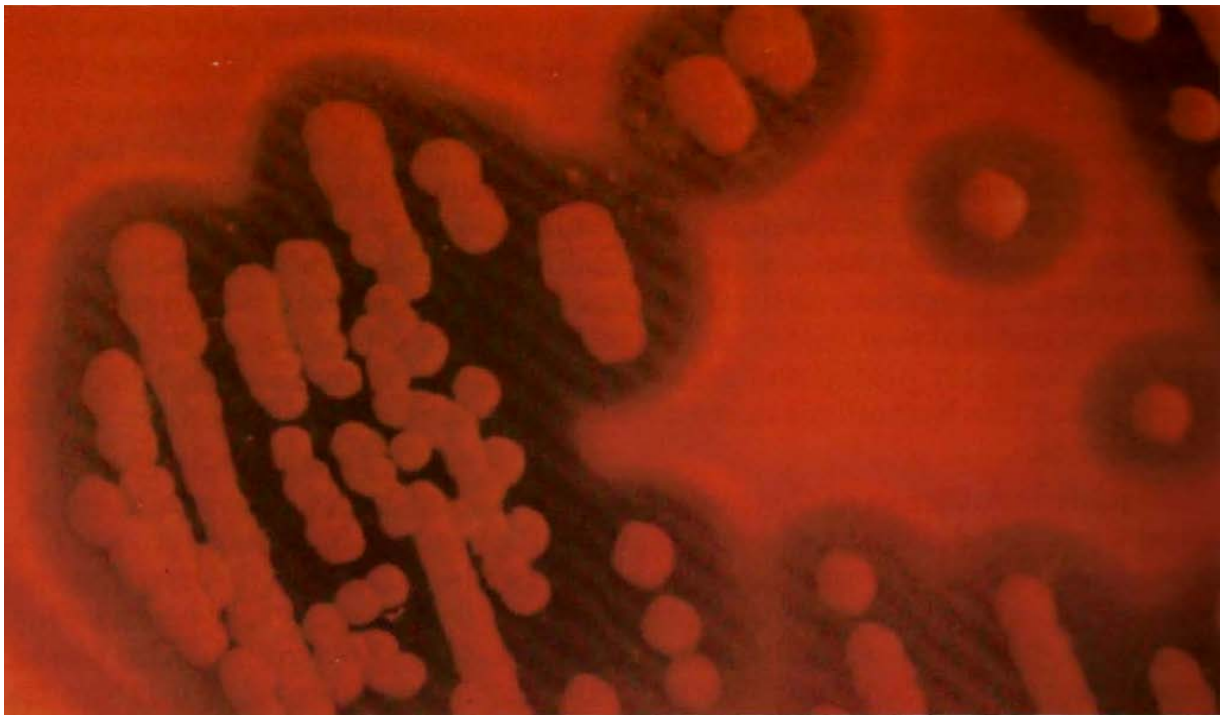

Figure VI-9. A hemolytic strain of *V. cholerae* on a sheep blood agar plate.

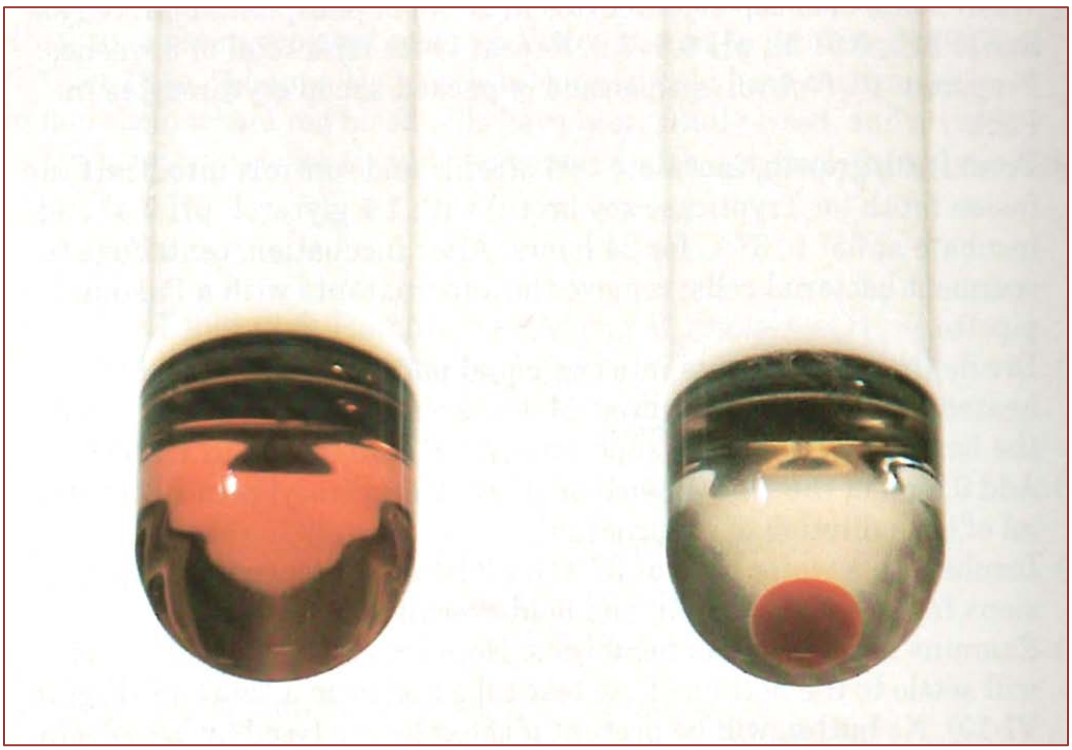

Figure VI-10. Tube hemolysis, shown in the tube on the left, is demonstrated by the absence of a "button" of sedimented cells and the presence of free hemoglobin in the supernatant.

Table VI-6. Differentiation of classical El Tor biotypes of *V. cholerae* serogroup O1

	Reaction	
Property	Classical	El Tor
Voges-Proskauer (modified with 1% NaCl)	-	+
Zone around polymyxin B (50 U)	+	-
Agglutination of chicken erythrocytes	-	+
Lysis by bacteriophage: Classical IV El Tor V.	+ -	- +

D. Tests for Determining Biotypes of *V. cholerae* O1

The differentiation of *V. cholerae* O1 into classical and El Tor biotypes is not necessary for control or treatment of patients, but may be of public health or epidemiologic importance in helping identify the source of the infection, particularly when cholera is first isolated in a country or geographic area. Only limited numbers of carefully selected isolates should be biotyped. Biotyping is not appropriate for *V. cholerae* non-O1, and the tests can give atypical results for nontoxigenic *V. cholerae* O1. The tests shown in Table VI-6 are commonly used in determining the biotype of *V. cholerae* O1. At least two or more of these tests should be used to determine biotype, since results can vary for individual isolates.
The El Tor biotype is currently predominant throughout the world and is the only biotype that has been isolated in the Western Hemisphere. The classical biotype is seen only rarely in most places, with the exception of Bangladesh.

1. Voges-Proskauer test

The Voges-Proskauer test has been used to differentiate between the El Tor and classical biotype of *V. cholerae* O1. Classical biotypes usually give negative results; El Tor isolates are generally positive.

2. Polymyxin B sensitivity

Sensitivity to the antimicrobial agent polymyxin B has been used to differentiate the biotypes of *V. cholerae* O1 (see Section E in this chapter for a description of antimicrobial susceptibility

testing procedures). A 50-unit polymyxin B disk (Mast Diagnostics, Merseyside, U.K.) is used for this test, and known strains of classical and El Tor biotypes are used as controls. The El Tor biotype is usually resistant to this concentration of polymyxin B and will not give a zone of inhibition (Figure VI-11). If there is any doubt about the result of this test, other biotyping tests should be performed or the isolate should be sent to a reference laboratory for confirmation. Classical strains are usually sensitive to polymyxin B and will give a zone of inhibition. Because the zone size is not important, any zone is interpreted as a positive result.

3. Hemagglutination (direct test)

Fresh chicken or sheep red blood cells can be used for this assay. A 2.5% (vol/vol) suspension of washed (3 times) and packed (by centrifugation) cells is made in normal saline after the final wash. A large loopful of the red cell suspension is placed on a glass slide. A small portion of the growth from a nonselective agar slant is added to the red cells with a needle or loop, and is mixed well. In a positive test, agglutination of the red cells occurs within 30 to 60 seconds (Figure VI-12). Hemagglutinating (El Tor) and nonhemagglutinating (classical) control strains should be used with each new suspension of red cells. Strains of classical *V. cholerae* O1 that have aged in the laboratory or have undergone repeated passage in broth may cause hemagglutination and should not be used as controls.

4. Bacteriophage susceptibility

Biotype may be determined by the susceptibility of an isolate of *V. cholerae* serogroup O1 to a specific bacteriophage. Classical strains of *V. cholerae* O1 are sensitive to cholera bacteriophage “Classical IV”; El Tor isolates are susceptible to bacteriophage “El Tor 5.” Although these tests are very reliable, the propagation, storage, and use of bacteriophage is technically demanding and is usually performed in only a few reference laboratories. If biotype determination by bacteriophage susceptibility is needed, it should be performed by a laboratory that uses this method routinely.

The use of bacteriophage in biotyping of *V. cholerae* O1 is briefly described as follows. The isolate to be tested is grown overnight in pure culture on a noninhibitory medium. From the overnight growth, brain heart infusion broth (or Trypticase soy broth) is inoculated and incubated for 6 hours at 35˚ to 37˚C. A lawn of bacteria in log-phase growth (OD=0.1) is then seeded onto the surface of a brain heart infusion agar plate by dipping a cotton swab into the 6-hour broth and lightly inoculating (swabbing) the entire surface of the plate. Positive and negative control strains should also be included. A drop of the bacteriophage diluted to routine test dilution (a measure of concentration of active bacteriophage particles) is applied to the bacterial lawn. The

plate is incubated overnight and read the next day. If the bacteria are susceptible to the bacteriophage they will be lysed, and there will be a zone of lysis in the bacterial lawn.

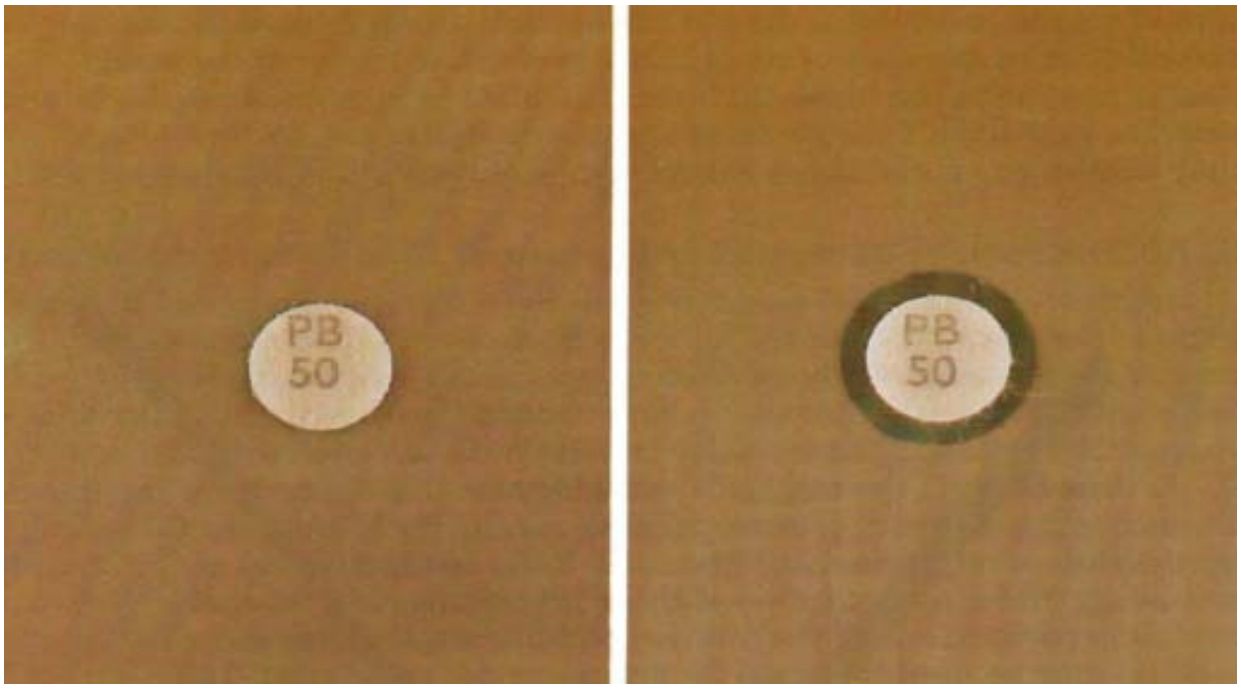

Figure VI-11. The El Tor strain of *V. cholerae* O1 on the left is resistant to the action of the antimicrobial agent polymyxin B (50 unit disk). A classical strain of *V. cholerae* O1, on the right, shows a characteristic zone of inhibition.

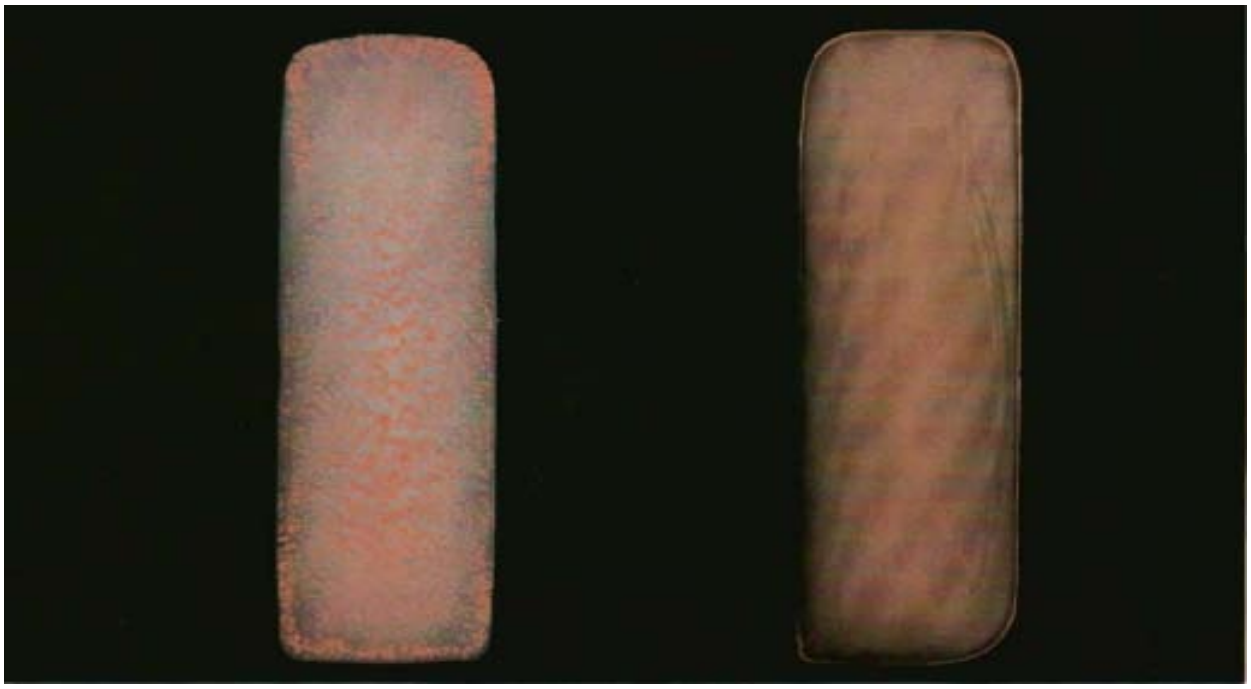

Figure VI-12. *V. cholerae* O1 biotype El Tor agglutinates sheep red blood cells as shown on the left. The classical biotype of *V. cholerae* O1 on the right does not agglutinate red blood cells.

E. Antimicrobial Susceptibility Testing (Agar Disk Diffusion Method)

The disk diffusion method presented here has been carefully standardized by the National Committee for Clinical Laboratory Standards (NCCLS) 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 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 susceptibility testing.

1. Special Considerations for Susceptibility Testing of *V. cholerae*

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 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. Antimicrobial agents suggested for use in susceptibility testing of *V. cholerae* are listed in Table VI-7 below.

Table VI-7. Antimicrobial agents suggested for use in susceptibility testing of *V. cholerae* O1 and O139

Trimethoprim-sulfamethoxazole
Choloramphenicol
Furazolidone
Tetracycline[a]

[a] The results from the tetracycline disk are used to predict susceptibility to doxycycline also.

Testing *V. cholerae* against certain drugs may yield misleading results when in vitro results do not correlate with in vivo activity. 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 only for ampicillin, chloramphenicol, sulfonamides, tetracycline and trimethoprim-sulfamethoxazole. It has been determined that disk diffusion results are not accurate for *V. cholerae* when testing erythromycin and doxycycline, and these agents should not be tested by this method. The results from the tetracycline disk should be used to predict susceptibility 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, had not been validated. Until interpretive criteria have been established for *V. cholerae*, disk diffusion may be used to screen for resistance to ciprofloxacin, using interpretive criteria for the *Enterobacteriaceae* as tentative zone size standards. Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* (see Table VI-8). When screening with the disk diffusion method for these agents, results should be interpreted with caution. If zone sizes for these drugs fall within the intermediate range, the organism should be considered possibly resistant.

2. Procedure for Agar Disk Diffusion

Mueller-Hinton susceptibility test agar

Mueller-Hinton agar medium is the only 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 Mueller-Hinton is prepared can affect disk diffusion test results, it is very important to refer to Section 3 below for instructions on preparation and quality control of this medium.

McFarland turbidity standard

A McFarland 0.5 standard should be prepared and quality controlled prior to beginning susceptibility testing (see Section 3). If tightly sealed to prevent evaporation and stored in the dark, the standard can be stored for up to 6 months. The McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test.

Preparation of inoculum

Each culture to be tested should be streaked onto a noninhibitory agar medium (blood agar, brain heart infusion agar, or tryptone soy agar) 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 (see Section 3) or nonselective broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland 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. The turbidity standard should be agitated on a vortex mirror immediately prior to use. If the bacterial suspension does not appear to be the same density as the McFarland standard 0.5, 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. Four to five colonies are picked from overnight growth on agar and inoculated into broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth). Incubate the broth at 35˚C until turbid, and then adjust the turbidity to 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. Finally, swab all around the edge of the agar surface.

Antimicrobial disks

The working supply of antimicrobial disks should be stored in the 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 disk. 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, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. In general, place no more than 12 disks on a 150-mm plate and no more than 4 disks on a 100mm plate. This prevents overlapping of the zones of inhibition and possible 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 to 18 hours. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) and record it in millimeters. The measurements can be made with 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 (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 (see Table VI-8), 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. The distance from the colony(ies) closest to the disk to the center of the disk should be measured and then doubled to obtain a diameter. The diameter of the outer clear zone should be recorded as well and interpretation recorded for each diameter. The colony(ies) inside the zone should be picked, re-isolated, re-identified, and retested in the disk diffusion test to confirm the previous results. The presence of colonies within a zone of inhibition may predict eventual resistance to that agent.

Quality control

To verify that susceptibility test results are accurate, it is important to include at least on control organism (ATCC 25922 is the *E. coli* control strain used when testing *Enterobacteriaceae* and *V. cholerae*) with each test. Zone diameters obtained for ATCC 25922). If zones produced by the control strain are out of expected ranges, the laboratorian should consider possible sources of error.

Susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be 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 to 4 mm or pH is not between 7.2 and 7.4, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

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

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.

As mentioned above, testing some bacteria against certain antimicrobial agents may yield misleading results because these in vitro results do not necessarily correlate with in vivo activity. Examples include erythromycin tested against *V. cholerae* (see section 1).

3. Preparation and Quality Control of Media and Reagents

Mueller-Hinton agar

[Note: Several commercial formulations of Mueller-Hinton agar are available. This medium should not be prepared from individual ingredients because this can diminish the quality. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

Follow manufacturer's instructions to prepare medium. After autoclaving, cool medium to 50˚C. Measure 60 to 70 ml of medium per plate into 15 x 150-mm plates, or measure 25 to 30 ml per plate into 15 x 100 mm plates. Agar should be poured into flat-bottom glass or petri dishes on a level pouring surface to a uniform depth of 4 mm. Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.

Freshly prepared plates may be used the same day or store in a refrigerator (2˚ to 8˚C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be place in an incubator (35 to 37˚C) until the moisture evaporates (usually 10 to 30 minutes). Do not leave lids ajar because the medium is easily contaminated.

Each new lot should be quality controlled before use by testing the *E. coli* ATCC 25922 and/or Staphylococcus aureus ATCC 25923 standard strains. These standard strains are used with every test run for Enterobacteriaceae and gram-positive aerobes, respectively. The pH of each new lot of Mueller-Hinton should be between 7.2 to 7.4. If outside the range, the pH medium should not be adjusted by the addition of acid or base; the batch of plates should be discarded and a new batch of plates prepared. If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory.

Turbidity standards (McFarland)

McFarland 0.5 turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22˚ to 25˚C). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube.

The accuracy of the density of a prepared McFarland standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1. Alternately, the accuracy of the McFarland standard may be verified by adjusting a suspension of a control strain (e.g., *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts. The adjusted suspension should give a count of 10^8 colony forming units/ml.

Physiological saline

NaCl	8.5g
Distilled water	1 liter

Dissolve NaCl in water, heating if necessary. May be sterilized by autoclaving or membrane filtration. Store at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

References

1. Jorgensen JH, Turnidge JD, Washington JA. Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of clinical microbiology, 7th ed., Washington, DC: ASM Press; 1999: 1526-1543.

2. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; ninth informational supplement. Wayne, Pennsylvania: NCCLS; 1999: document M100-S9, Vol. 19. No. 1, Table 21.

Table VI-8. Zone size interpretative standards for Vibrio cholerae for selected antimicrobial disks

Antimicrobial Agent	Disk Potency (µg)	Resistant	Zone diameter (mm) Intermediate	Susceptible	Zone diameter limits (mm) for E. coli ATCC 25922
Choloramphenicol[a,b]	30	≤12	13-17	≥18	21-27
Ampicillin[a]	10	≤13	14-16	≥17	16-22
Furazolidone[c] for *V. cholerae*	100	<18	-	≥18	22-26[c]
Trimethoprim-sulfamethoxazole[a]	1.25/ 23.75	≤	11-15	≥16	24-32
Tetracycline[a]	30	≤14	15-18	≥19	18-25
Ciprofloxacin[a,d]	5	≤15	16-20	≥21	30-40
Nalidixic acid[c] *for V. cholerae*	30	<19	-	≥19	22-28

[a]Source: National Committee on Clinical Laboratory Standards (NCCLS), 1998.
[b]Use these interpretive standards with caution as the disk diffusion test may misclassify many organisms (high minor error rate)
[c]Proposed interpretive criteria based on multi-laboratory studies. Criteria have not been established for *V. cholerae* by NCCLS.
[d]These zone sizes are valid for interpreting disk diffusion results for *Enterobacteriaceae*. However, Zone sizes for *V. cholerae* have not been established by NCCLS.