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 VI-1). 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 added 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 the 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 nitrite	Myo-inositol fermentation	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase
Group 1								
V. cholerae	+	+	+	+	-	-	+	+
V. mimicus	+	+	+	+	-	-	+	+
Group 2								
V. metschnikovii	-	+	-	-	V	V	V	-
Group 3								
V. cincinnatiensis	-	+	+	+	+	-	V	-
Group 4								
V. hollisae	-	+	+	+	-	-	-	-
Group 5								
V. damsela	-	+	+	+	-	+	V	-
V. fluvialis	-	+	+	+	-	+	-	-
V. furnissii	-	+	+	+	-	+	-	-
Group 6								
V. alginolyticus	-	+	+	+	-	-	+	V
V. parahaemolyticus	-	+	+	+	-	-	+	+
V. vulnificus	-	+	+	+	-	-	+	V
V. carchariae	-	+	+	+	-	-	+	-

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

[a] Difco Laboratories, Detroit, MI.

Table VI-2. Characteristics of *Vibrio cholerae*

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 produce other toxins

[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 agglutinate 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 O1 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 a 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 10 µl 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).

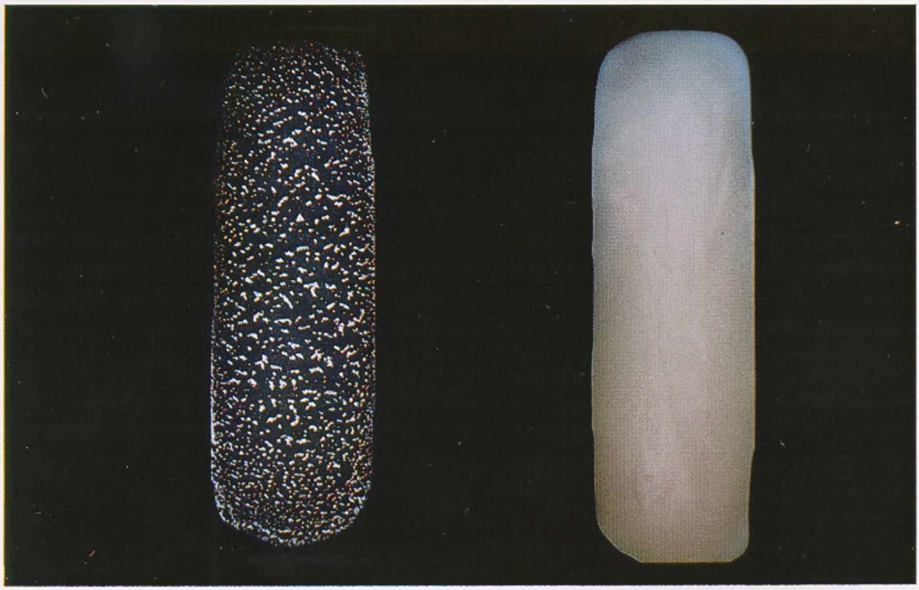

Figure VI-1. Antisera to the O1 serogroup of *V. cholerae* will agglutinate homologous organisms (left). A normal serum or saline control (right) does not show agglutination.

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

Test	% positive
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 0% 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 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-fer-

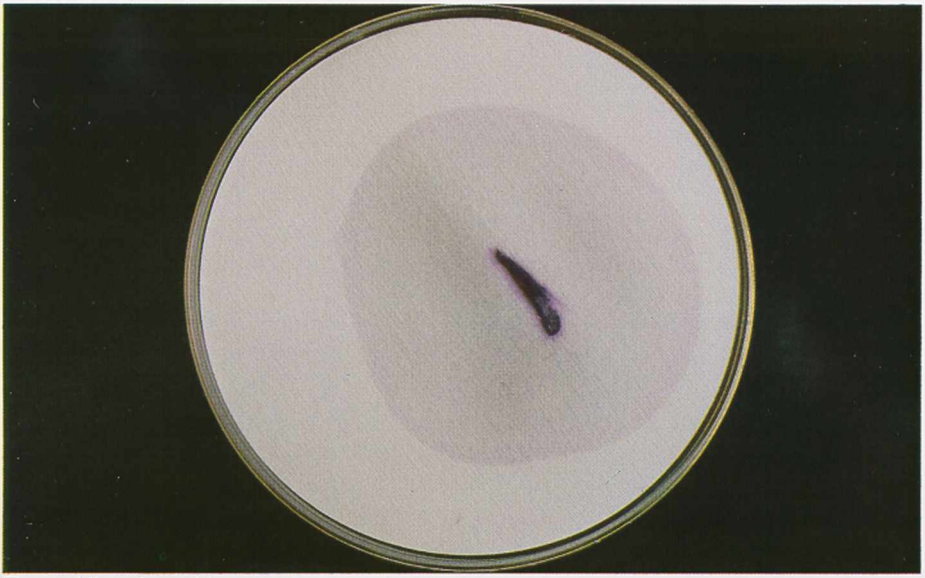

Figure VI-2. A positive oxidase test (as shown here) results in the development of a dark purple color within 10 seconds. *V. cholerae* is oxidase-positive, which differentiates it from oxidase-negative organisms such as the *Enterobacteriaceae*.

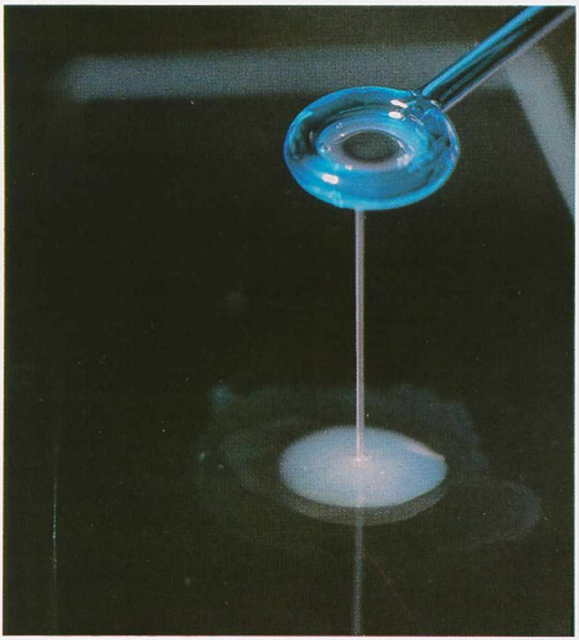

Figure VI-3. A positive string test, shown here with *V. cholerae*, is a rapid and simple method for distinguishing between the genus *Vibrio* (mostly positive) and *Aeromonas* (nearly always negative).

menting *Enterobacteriaceae* (K/A, no gas, no H_2S) (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 TSI 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 and 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 and 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 decorboxylase.

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 alka-

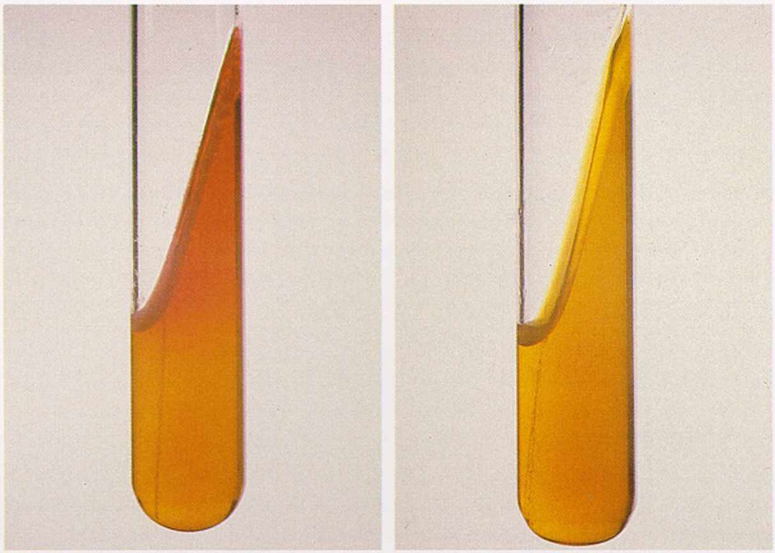

Figure VI-4. Reactions of *V. cholerae* in Kligler's iron agar (left) and triple sugar iron agar (right).

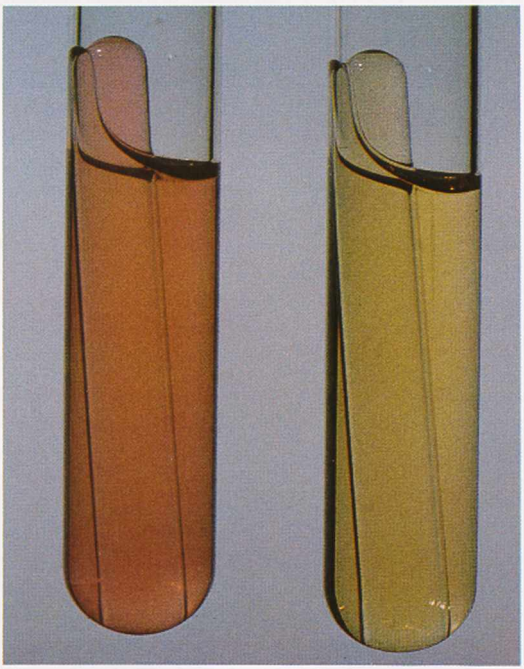

Figure VI-5. With Andrade indicator in the carbohydrate medium, a pink color develops if fermentation has occurred, while negative reactions appear yellow.

line slant (purple) and an acid butt (yellow). *V. cholerae* gives a K/K reaction in LIA (lysine positive) but produces a K/A reaction (arginine negative) in AGS.

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).

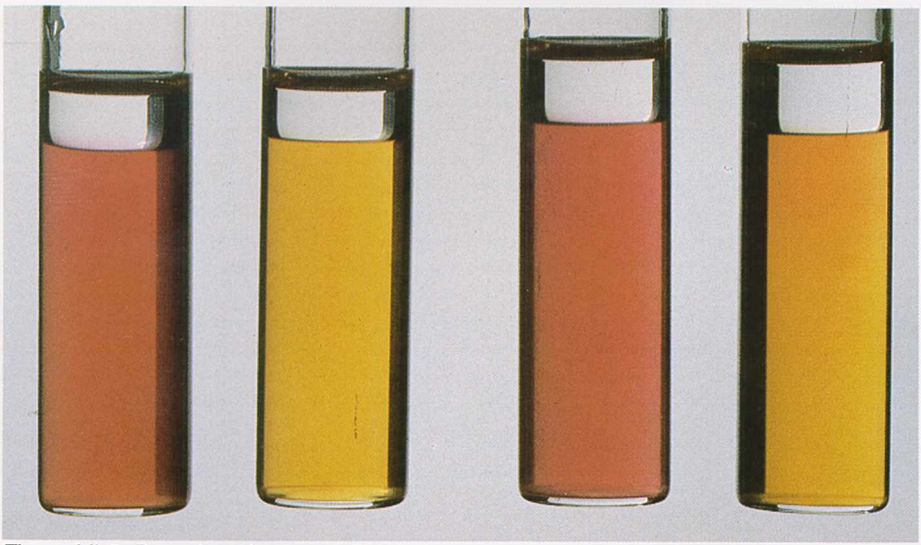

Figure VI-6. Decarboxylase and dihydrolase reactions for *V. cholerae* are, from left to right, lysine (+), arginine (-), ornithine (+), and control (-).

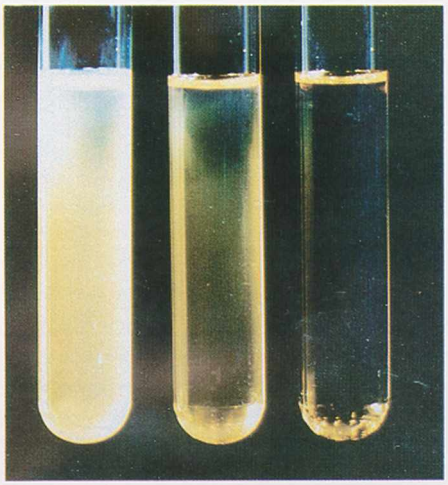

Figure VI-7. *V. cholerae* grows in the absence of NaCl (tube B), but growth is stimulated by the addition of 1% NaCl (tube A). Tube C, 0% NaCl, inoculated with *V. parahaemolyticus*, shows no growth.

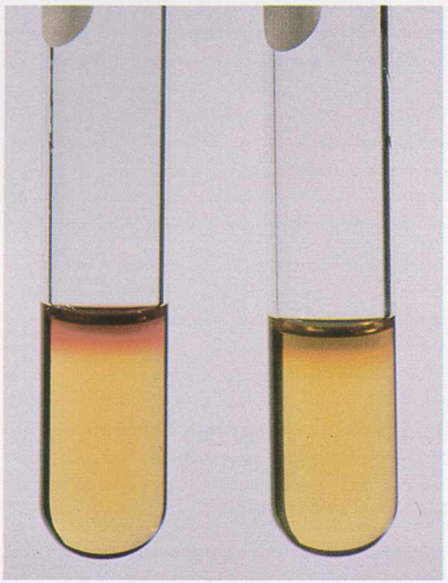

Figure VI-8. *V. cholerae* produces acetoin, which is detected in the Voges-Proskauer test, giving a red (positive) reaction (left). A negative reaction is on the right.

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 150 μg 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 con-

Table VI-5. Hemolytic activity of *V. cholerae* 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.

trol strain (Figure VI-9). Strains that give incomplete hemolysis (incomplete clearing of the red blood cells) should not be reported as hemolytic.

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 described 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) Divide 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.

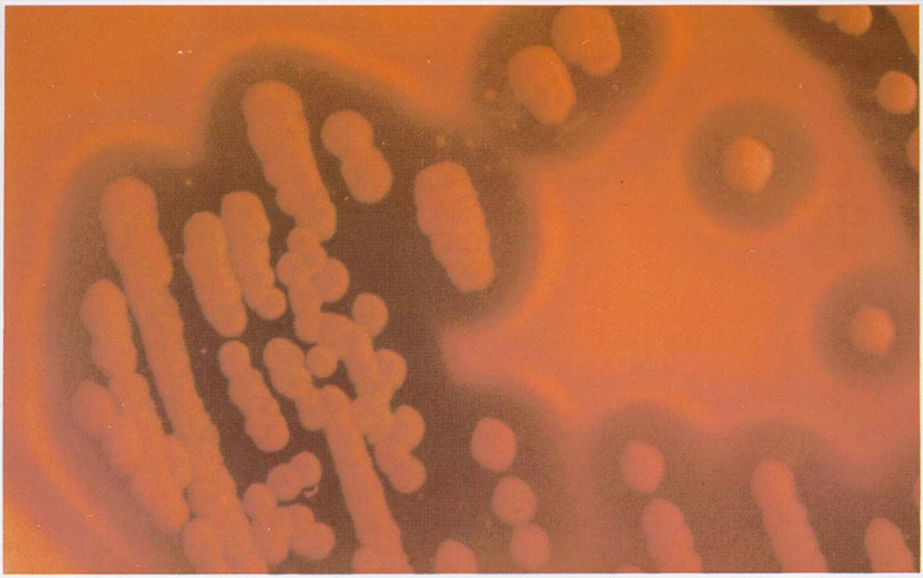

Figure VI-9. A hemolytic strain of *V. cholerae* on a sheep blood agar plate.

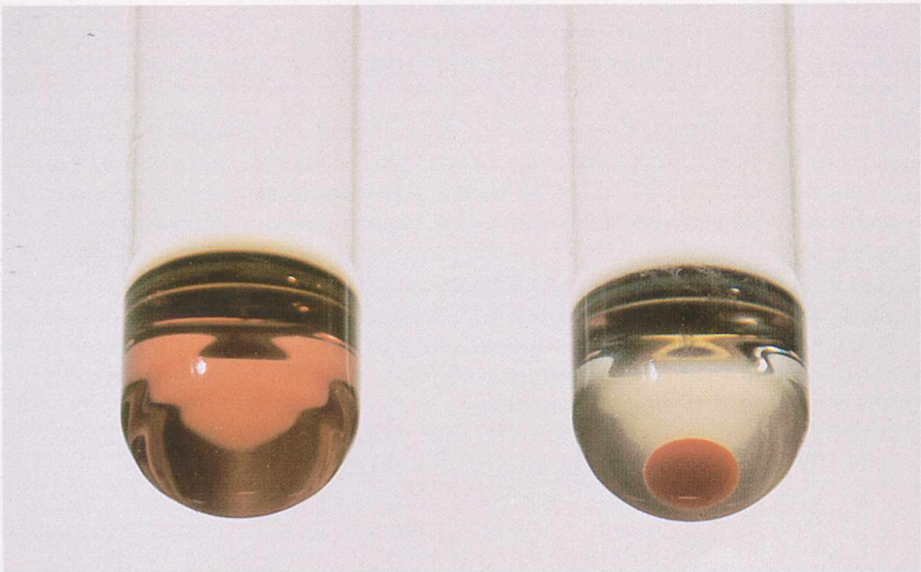

Figure VI-10. Tube hemolysis, shown in the tube on the left, is demonstrated by the absence of a "button" of sedimented cells and the presence of free hemoglobin in the supernatant.

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

Property	Reaction	
	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	-	+

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.

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 biotypes 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 the 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

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.

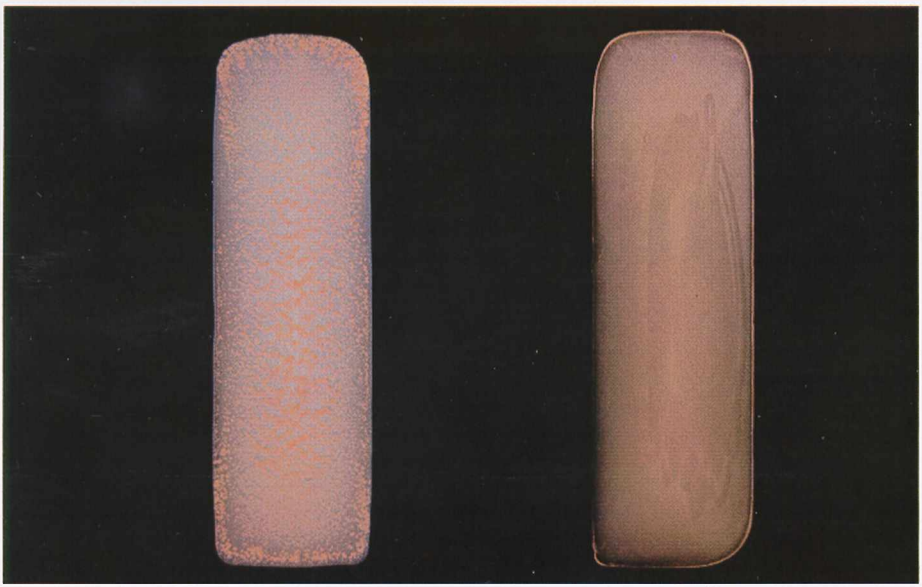

Figure VI-12. *V. cholerae* O1 biotype El Tor agglutinates sheep red blood cells as shown on the left. The classical biotype of *V. cholerae* O1 on the right does not agglutinate red blood cells.

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.

E. Antimicrobial Susceptibility Test (Agar Disk Diffusion Method)

Because antimicrobial resistance has been a growing problem in some parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be monitored periodically. Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, interpretive criteria have not been established for *V. cholerae* O1, and the method's reliability for this organism is unknown. To ensure the accuracy of susceptibility results for *V. cholerae* O1, agar or broth dilution methods should be used. Refer to the Manual of Clinical Microbiology (6) for a description of these procedures. If a laboratory cannot routinely perform one of the dilution techniques, the disk diffusion method may be used to screen for antimicrobial resistance. Table VI-7 lists the interpretive criteria for the antimicrobial agents that are currently recommended by the World Health Organization for treatment of cholera (tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, and chloramphenicol) as well as some other commonly used antimicrobial drugs. These criteria, which have been standardized for the *Enterobacteriaceae*, may be used as tentative zone size standards for screening for antimicrobial resistances in *V. cholerae* O1 until interpretive criteria have been validated. When the disk diffusion method is used for screening, any isolates that fall within the intermediate or resistant ranges should be tested with a dilution method to obtain the minimum inhibitory concentration of the drug in question.

Procedure for agar disk diffusion

For a more complete description of this method, refer to the Manual of Clinical Microbiology (4).

Mueller Hinton agar medium should be prepared and autoclaved according to the manufacturer's directions. After the agar has been cooled to approximately 50°C in a water bath, the medium should be poured into 15 x 150-mm petri dishes to a depth of 4 mm (approximately 60 to 70 ml per plate). Dry the plates in an incubator for 10 to 30 minutes before use.

Prepare a 0.5 McFarland turbidity standard by adding 0.5 ml of 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% sulfuric acid. The turbidity standard should be in a test tube identical to the one to be used to grow the test organism in broth. The McFarland standard should be sealed with wax, Parafilm, or some other means to prevent evaporation, and may be stored for up to 6 months at room temperature (22° to 25°C) in the dark.

Table VI-7. Zone size interpretative standards for the *Enterobacteriaceae* for selected antimicrobial disks (not validated for *V. cholerae* O1)

Antimicrobial agent	Disk potency (μg)	Zone diameter (mm)[a]			Zone diameter limits (mm) for *E. coli* ATCC 25922
		Resistant	Intermediate	Sensitive	
Chloramphenicol	30	≤12	13-17	≥18	21-27
Doxycycline	30	≤12	13-15	≥16	18-24
Erythromycin	15	≤13	14-22	≥23	8-14[b]
Furazolidone	100	≤13	14-17	≥18	22-26[c]
Trimethoprim-sulfamethoxazole	1.25/23.75	≤10	11-15	≥16	24-32
Tetracycline	30	≤14	15-18	≥19	18-25
Ciprofloxacin	5	≤15	16-20	≥21	30-40
Nalidixic acid	30	≤13	14-18	≥19	22-28

[a] Source: National Committee on Clinical Laboratory Standards (NCCLS), 1992. Zone sizes for *V. cholerae* have not been established by NCCLS.

[b] Source: World Health Organization.

[c] Source: Manufacturer.

Each culture to be tested should be streaked onto a noninhibitory agar medium (blood agar, brain heart infusion agar, or Trypticase soy agar) to obtain isolated colonies. After incubation at 35° to 37°C overnight, select well-isolated colonies with an inoculating needle or loop and transfer the growth to a tube of sterile broth (Mueller-Hinton broth, heart infusion broth, or Trypticase soy broth). Emulsify a sufficient quantity of bacterial growth in the broth so that the turbidity approximates that of 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. If necessary, turbidity can be reduced by adding sterile broth. Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile nontoxic 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 3 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.

The working supply of antimicrobial disks should be kept in the refrigerator. After removal from the refrigerator, the containers should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation that occurs when warm air reaches the cold container. 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. The antimicrobial disks should be applied to the plates as soon as possible, but no longer than 15 minutes after inoculation. After the disks are placed on the plate, the plate should be placed in an incubator at 35°C for 16 to 18 hours.

After overnight incubation, the diameter of the zones of complete inhibition (including the diameter of the disk) is measured and recorded 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-7), and recorded as susceptible, intermediate, or resistant to each drug tested.

Susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. To obtain reliable test results, it is important to include control organisms with each test and to follow the procedure precisely (see Table VI-7 for diameters of the zones of inhibition for the quality control strain). A decrease in potency of the disks after storage may be indicated by a decrease in the size of the inhibition zone around the control strain.

References

1. Kelly MT, Hickman-Brenner FW, Farmer JJ III. *Vibrio*. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of Clinical Microbiology. 5th ed. Washington, D.C.: American Society for Microbiology, 1991: 384-95.
2. Barrett TJ, Blake PA. Epidemiological usefulness of changes in hemolytic activity of *Vibrio cholerae* biotype El Tor during the seventh pandemic. J Clin Microbiol 1981;13:126-9.
3. World Health Organization. Manual for Laboratory Investigations of Acute Enteric Infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.
4. Barry AL, Thornsberry C. Susceptibility tests: diffusion test procedures. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of Clinical Microbiology. 5th ed. Washington, D.C.: American Society for Microbiology, 1991: 1117-25.
5. Feeley JC. Classification of *Vibrio cholerae* (*Vibrio comma*), including El Tor vibrios, by infrasubspecific characteristics. J Bacteriol 1965;89: 665-70.
6. Sahm DF, Washington JA II. Antibacterial susceptibility tests: dilution methods. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of Clinical Microbiology. 5th ed. Washington, D.C.: American Society for Microbiology, 1991: 1105-16.