

Chapter 6

Isolation and Identification of *Vibrio cholerae* Serogroups O1 and O139

Isolation and identification of *V. cholerae* serogroups O1 and O139 can be greatly enhanced when optimal laboratory media and techniques are employed. The methods presented here are intended to be economical and to offer laboratorians some flexibility in choice of protocol and media. Laboratories that do not have sufficient resources to adopt the methods described in this chapter should consider sending the specimens or isolates to other laboratory facilities that routinely perform these procedures.

A. Isolation Methods

Before 1992, of the more than 150 serogroups of *Vibrio cholerae* that have been reported, only the O1 serogroup was associated with epidemic and pandemic cholera. However in late 1992 and early 1993, large outbreaks of cholera due to a newly described serogroup, O139, were reported in India and Bangladesh. This strain, like serogroup O1 *V. cholerae*, produces cholera enterotoxin. 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. Annex A lists diagnostic supplies necessary for laboratory confirmation and antimicrobial susceptibility testing of *V. cholerae*.

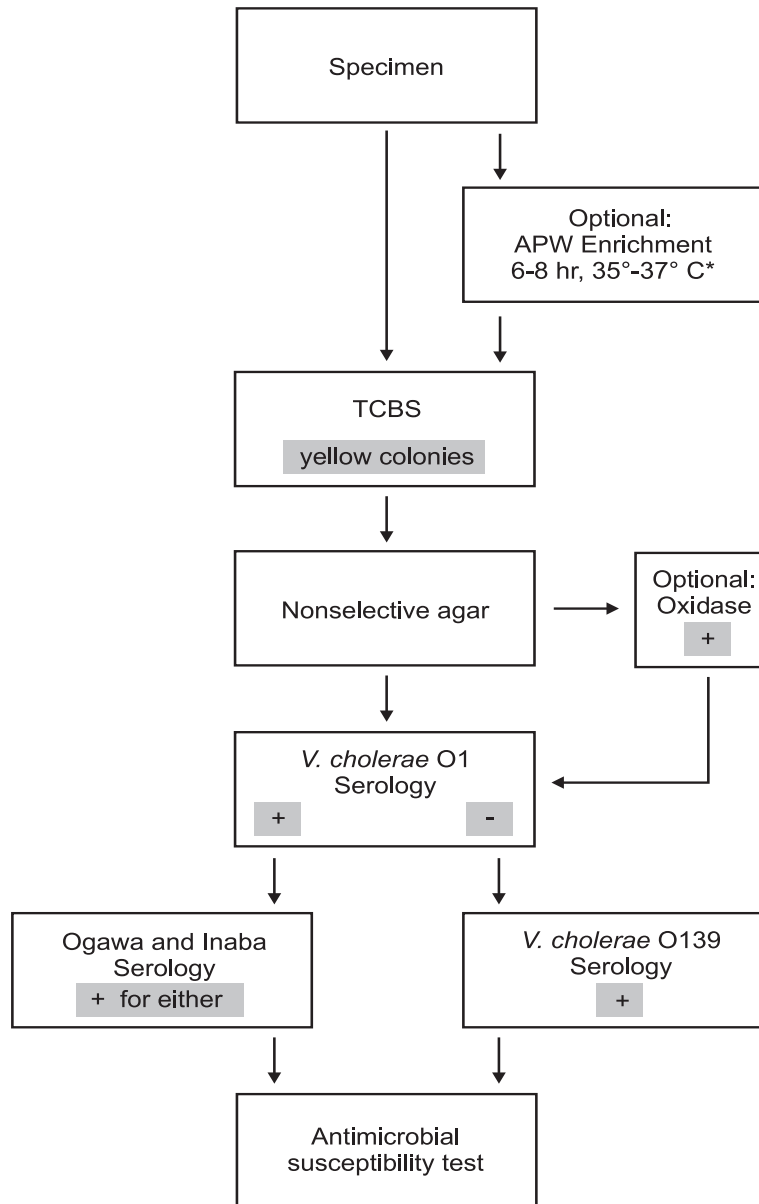
Although *V. cholerae* will grow on a variety of commonly used agar media, isolation from fecal specimens is more easily accomplished with specialized media. Alkaline peptone water (APW) is recommended as an enrichment broth, and thiosulfate citrate bile salts sucrose agar (TCBS) is the selective agar medium of choice (Figure 6-1). In certain instances (for example, when the patient is in very early stages of illness), it may not be necessary to enrich specimens or use selective plating media. However, enrichment broth and a selective plating medium should always be used with convalescent patients, suspected asymptomatic infections, environmental specimens, and whenever high numbers of competing organisms are likely to be present in the specimen.

Refer to Section C, “Media and Reagents for *V. cholerae*,” before preparing any of these media because incorrect preparation can affect the reactions of organisms in these tests. Chapter 11 discusses methods for quality control of selective media and antisera.

1. Enrichment in alkaline peptone water

Enrichment in APW can enhance the isolation of *V. cholerae* when few organisms are present, as in specimens from convalescent patients and asymptomatic carriers. *Vibrio* spp. grow very rapidly in APW, and at 6 to 8 hours will be present in greater numbers than non-*Vibrio* organisms.

Isolation and Identification of *Vibrio cholerae* Serogroups O1 and O139



* If the 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 then streak to TCBS

Figure 6-1. Procedure for recovery of *Vibrio cholerae* O1 and O139 from fecal specimens

APW can be inoculated with liquid stool, fecal suspension, or a rectal swab. The stool inoculum should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35° to 37°C for 6 to 8 hours. After incubation, subculture to TCBS should be made with one to two loopfuls of APW from the surface and topmost portion of the broth, since vibrios preferentially grow in this area. Do not shake or mix the tube before subculturing. If the broth cannot be plated after 6 to 8 hours of incubation, subculture a loopful at 18 hours to a fresh tube of APW. Subculture this second tube to TCBS agar after 6 to 8 hours of incubation (Figure 6-1).

2. Isolation from TCBS selective agar

TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective (see Section C). Growth on this medium is not suitable for direct testing with *V. cholerae* antisera.

Inoculation of TCBS

Figure 6-1 outlines the procedure for isolation of *V. cholerae* from fecal specimens. Inoculate the TCBS plate as described in Chapter 4 (Figure 4-2). After 18 to 24 hours' incubation at 35° to 37°C, the amount and type of growth (e.g., sucrose-fermenting or sucrose-nonfermenting) on the TCBS plate should be recorded on data sheets (Figure 6-2). Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2 to 4 mm in diameter (Figure 6-3). The yellow color is caused by the fermentation of sucrose in the medium. Sucrose-nonfermenting organisms, such as *V. parahaemolyticus*, produce green to blue-green colonies.

Isolation of suspected V. cholerae

Carefully select at least one of each type of sucrose-fermenting colony from the TCBS plate to inoculate a heart infusion agar (HIA) slant or another nonselective medium. Do not use nutrient agar because it has no added salt and does not allow optimal growth of *V. cholerae*. Using an inoculating needle, lightly touch only the very center of the colony. Do not take the whole colony or go through the colony and touch the surface of the plate. This is to avoid picking 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.

Incubate the HIA slants at 35° to 37°C for up to 24 hours; however, there may be sufficient growth at 6 hours for serologic testing to be done. Slide serology with polyvalent O1 and O139 antisera is sufficient for a presumptive identification (see section B below for a description of serologic identification).

Vibrio cholerae Worksheet

SPECIMEN NUMBER	MEDIA	SUC + ^a	SUC - ^b	COLONY	OPTIONAL			SLIDE SEROLOGY				IDENTIFICATION
					OXIDASE TEST	STRING TEST	GRAM STAIN OR WET MOUNT	PV O1	INABA	OGAWA	O139	
	DIRECT TCBS			T1								
				T2								
				T3								
	APW- TCBS			AT1								
				AT2								
				AT3								
	DIRECT TCBS			T1								
				T2								
				T3								
APW- TCBS			AT1									
			AT2									
			AT3									
	DIRECT TCBS			T1								
				T2								
				T3								
	APW- TCBS			AT1								
				AT2								
				AT3								

^aSUC + = Sucrose-positive colonies ^bSUC - = Sucrose-negative colonies

Figure 6-2. *Vibrio cholerae* worksheet



Figure 6-3. Growth of *V. cholerae* on TCBS

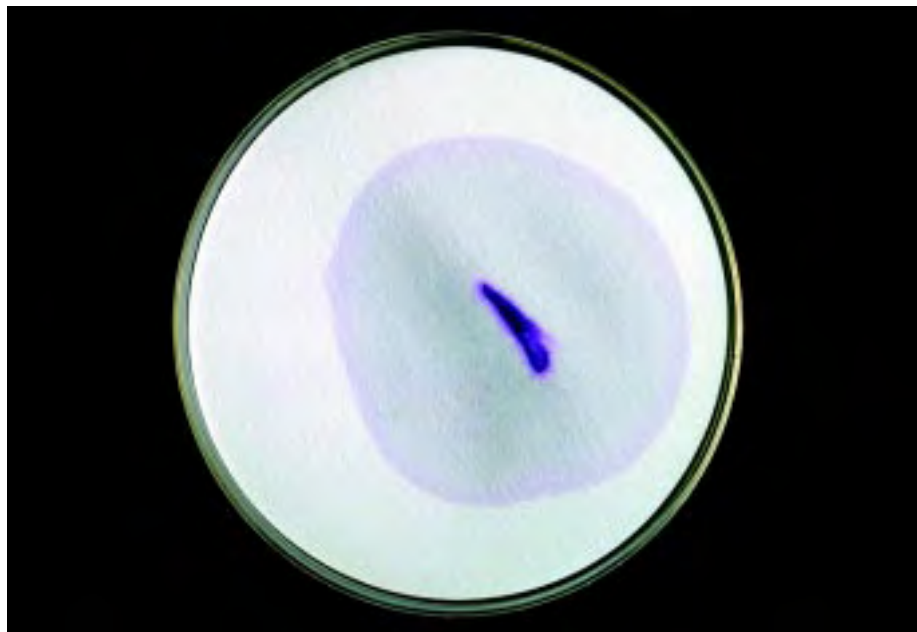


Figure 6-4. A positive oxidase test (shown here) results in the development of a dark purple color within 10 seconds. *V. cholerae* is oxidase positive.

3. Screening tests for suspected *V. cholerae* isolates

Generally for suspected *V. cholerae* isolates from fecal specimens, screening with biochemical tests prior to testing with O1 and O139 antisera is not necessary. However, if the supply of antisera is limited, the oxidase test may be useful for additional screening of isolates before testing with antisera.

Oxidase test

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate-containing medium. Do not use growth from TCBS agar because it may yield either false-negative or false-positive results. Place 2 to 3 drops of oxidase reagent (1% *N,N,N',N'*-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 immediately (Figure 6-4). Oxidase-negative organisms will remain colorless or will turn purple after 10 seconds. Color development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio* (including *V. cholerae*, Table 6-1), *Neisseria*, *Campylobacter*, *Aeromonas*, *Plesiomonas*, *Pseudomonas*, and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.

Table 6-1. Reactions of *V. cholerae* in screening tests

Screening test	<i>Vibrio cholerae</i> reactions
Oxidase test	Positive
String test	Positive
KIA	K/A, no gas produced (red slant/yellow butt) ^a
TSI	A/A, no gas produced (yellow slant/yellow butt) ^a
LIA	K/K, no gas produced (purple slant/purple butt) ^{a,b}
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 of the medium indicates that lysine was not decarboxylated.

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 6-1). The value of these tests should be assessed to determine their usefulness before they are applied routinely. See Section C for instructions on preparation of media and appropriate quality control strains.

String test

The string test, using fresh growth from nonselective agar, is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas* spp. 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 muroid “string” is formed when an inoculating loop is drawn slowly away from the suspension (Figure 6-5). *V. cholerae* (Table 6-1) strains are positive, whereas *Aeromonas* strains are usually negative. Other *Vibrio* spp. may give a positive or weak string test reaction.

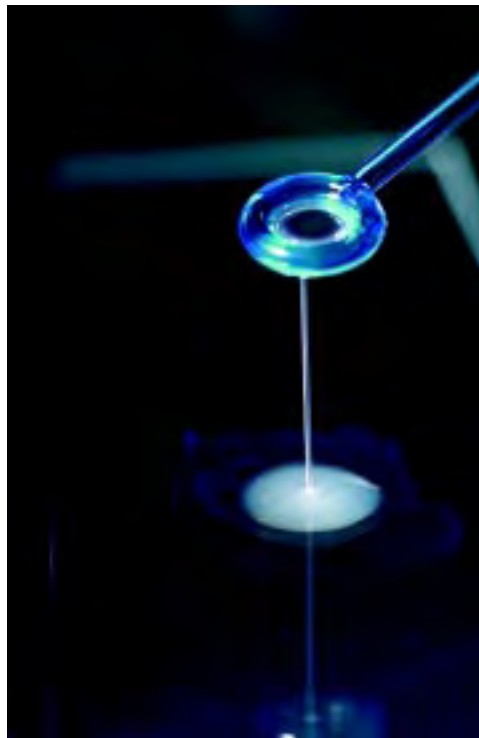


Figure 6-5. A positive string test with *V. cholerae*

Kligler iron agar and triple sugar iron agar

Kligler iron agar (KIA) and triple sugar iron agar (TSI) can be used to rule out *Pseudomonas* spp. and certain *Enterobacteriaceae*. The reactions of *V. cholerae* on KIA, which contains glucose and lactose, are similar to those of lactose-nonfermenting *Enterobacteriaceae* (alkaline (red) slant, acid (yellow) butt, no gas, no H₂S) (see Table 6-1 and Figure 6-6). However, on TSI, *V. cholerae*

strains produce an acid (yellow) slant and acid (yellow) butt, no gas, and no H₂S.

KIA or TSI slants are inoculated by stabbing the butt and streaking the surface of the medium. Incubate the slants at 35° to 37°C and examine 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, an inappropriate reaction will occur and the characteristic reactions of *V. cholerae* may not be exhibited.

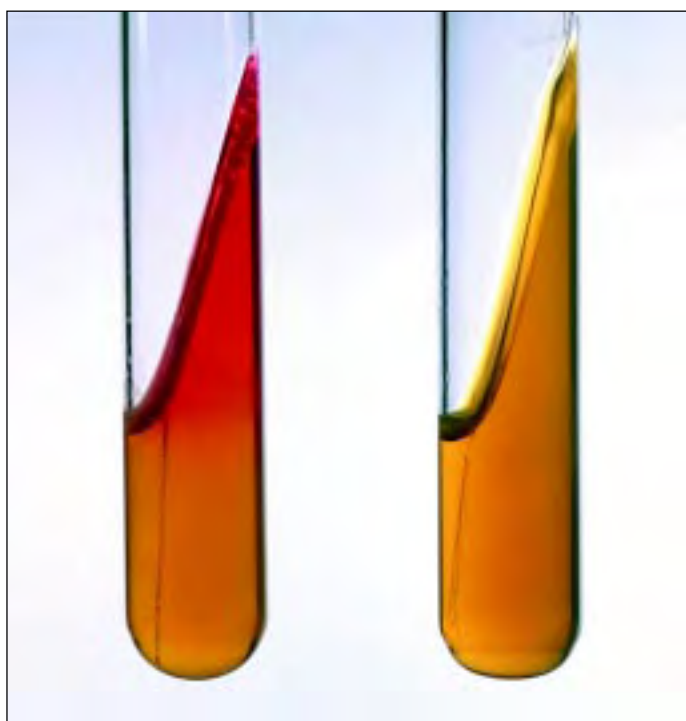


Figure 6-6. Reactions of *V. cholerae* in Kligler iron agar (left) and triple sugar iron agar (right)

Lysine iron agar

LIA is helpful for screening out *Aeromonas* and certain *Vibrio* spp., which, unlike *V. cholerae*, do not decarboxylate lysine. LIA is inoculated by stabbing the butt and streaking the slant. After incubation for 18 to 24 hours at 35° to 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 (see Chapter 4, Figure 4-11). Organisms without the enzyme

produce a yellow color (acid) 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 6-1). *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine.

It is important that KIA, TSI, and LIA 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. In LIA, the decarboxylation of lysine occurs only in anaerobic conditions and a false-negative reaction may result from insufficient medium in the tube (Section C).

Gram stain

Examining overnight growth from an HIA slant by Gram stain will demonstrate typical small, curved gram-negative rods (Table 6-1). Staining with crystal violet only is a more rapid technique and will still demonstrate the cell morphology typical of *Vibrio* spp.

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 (“shooting star”) motility (Table 6-1).

B. Serologic Identification of *V. cholerae* O1 and O139

1. 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 TCBS agar may result in false-negative reactions. Usually after 5 to 6 hours of incubation, growth on the surface of the slant is sufficient to perform slide serology 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, it may be reported as presumptive *V. cholerae* O1 or O139. Presumptive *V. cholerae* O1 isolates should be tested in monovalent Ogawa and Inaba antisera (see below). 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.

2. 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 (very rare). Serotype identification is based on agglutination in monovalent antisera to type-specific O antigens (see Table 6-2). 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.”

Refer to Chapter 11 for a discussion on quality control of antisera.

Table 6-2. Serotypes of *V. cholerae* serogroup O1

<i>V. cholerae</i> O1 serotype	Agglutination in absorbed antiserum	
	Ogawa antiserum	Inaba antiserum
Ogawa	+	-
Inaba	-	+
Hikojima	+	+

3. Slide agglutination procedures

Agglutination tests for *V. cholerae* somatic O antigens may be carried out in a petri dish or on a clean glass slide. Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of HIA, KIA, TSI, or other nonselective agar medium. Emulsify the growth in two small drops of physiological saline and mix thoroughly. Add a small drop of antiserum to one of the suspensions. 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 microliters (0.01 ml) can be used. An inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 4-12). Mix the suspension and antiserum well and then tilt the slide back and forth to observe for agglutination. If the reaction is positive, clumping will appear within 30 seconds to 1 minute. Examine the saline suspension carefully to ensure that it does not show clumping due to autoagglutination. If autoagglutination occurs, the culture is termed “rough” and cannot be serotyped.

4. 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. No serotypes have been identified in the O139 serogroup.

C. Media and Reagents for *V. cholerae*

1. Alkline peptone water

[Note: There are several different published formulations for this medium.]

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Add ingredients to the water and adjust to pH 8.5 with 3 N NaOH solution. Distribute and autoclave at 121°C for 15 minutes. Store at 4°C for up to 6 months making sure caps are tightly closed to prevent a drop in pH or evaporation.

When inoculated into APW for quality control, *V. cholerae* O1 should show good growth at 6 to 8 hours.

2. Kligler iron agar and triple sugar iron agar

[Note: There are several commercially available dehydrated formulations of KIA and TSI. These media can also be prepared from individual ingredients but there may be lot-to-lot variation.]

Prepare according to manufacturer's instructions. Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is 3 cm deep and the slant is 2 cm long. Tighten caps and store at 4°C for up to 6 months.

Each new lot should be quality controlled before use. *E. coli* should give an acid slant and butt, with production of gas but no H₂S. *S. flexneri* should give an alkaline slant, acid butt, without production of gas or H₂S [Note: some *S. flexneri* 6 strains produce gas].

3. Lysine iron agar

[Note: Several companies sell dehydrated LIA. LIA may also be prepared from individual ingredients but there may be lot-to-lot variation.]

Prepare medium according to manufacturer's instructions on the bottle. Dispense a quantity of medium in appropriate containers such that the volume of

medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is about 3 cm deep and the slant is about 2 cm long. When the agar is cooled and solidified, tighten caps and store at 4°C for up to 6 months.

Each new lot of dehydrated medium should be quality controlled before use. *S. flexneri* should produce an alkaline slant and an acid butt without production of H₂S. H₂S-producing *Salmonella* strains should produce an alkaline slant and an alkaline butt with blackening of the medium due to H₂S. *V. cholerae* strains are lysine-positive and will produce an alkaline reaction in the butt of the LIA.

4. Oxidase reagent

<i>N,N,N',N'</i> -Tetramethyl- <i>p</i> -phenylenediamine dihydrochloride	0.05 g
Distilled water	5.0 ml

Dissolve the reagent in purified water (do not heat to dissolve). Prepare fresh daily.

Positive and negative controls should be tested every time the reagent is prepared. *V. cholerae* is oxidase positive; *E. coli* is oxidase negative.

5. Sodium deoxycholate reagent (0.5%) for string test

Sodium deoxycholate	0.5 g
Sterile distilled water	100.0 ml

Add sterile distilled water to sodium deoxycholate and mix well. Store at room temperature for up to 6 months.

Each new batch should be quality controlled before use. A *V. cholerae* O1 strain should be used as positive control. *E. coli* may be used as a negative control.

6. Thiosulfate citrate bile salts sucrose agar

[Note: Several commercial brands of thiosulfate citrate bile salts sucrose agar (TCBS) agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.]

Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. Heat with agitation. Medium should be completely dissolved. Cool agar in a water bath until cool enough to pour (50° to 55°C). Pour into petri plates, leaving lids ajar about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 week.

Each new lot should be quality controlled before use since TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity. *V. cholerae* O1 should

show good growth of yellow colonies. *E. coli* should have none to poor growth of translucent colonies.

References

World Health Organization. Manual for the laboratory investigations of acute enteric infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.

McLaughlin JC. *Vibrio*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Tenover FC, ed. Manual of clinical microbiology. Washington, DC: ASM Press; 1995:465-476.

Centers for Disease Control and Prevention. Laboratory methods for the diagnosis of *Vibrio cholerae*. Atlanta, Georgia: CDC; 1994.

Kay, BA, Bopp CA, Wells JG. Isolation and identification of *Vibrio cholerae* O1 from fecal specimens. In: Wachsmuth IK, Blake PA, and Olsvik O., ed. *Vibrio cholerae* and cholera: molecular to global perspectives. Washington, DC: ASM Press; 1994: 3-26. Worksheet

Isolation and Identification of Vibrio cholerae Serogroups O1 and O139