Chapter 9. V. cholerae, V. parahaemolyticus, V. vulnificus, and Other Vibrio spp.

ELISA L. ELLIOT, CHARLES A. KAYSNER, and MARK L. TAMPLIN

The genus Vibrio includes Gram-negative, oxidase-positive (except two species), rod- or curved rod-shaped facultative anaerobes. Many Vibrio spp. are pathogenic to humans and have been implicated in foodborne disease (Table 1). Vibrio spp. other than V. cholerae and V. mimicus do not grow in media that lack added sodium chloride, and are referred to as "halophilic."

V. cholerae was first described as the cause of cholera by Pacini in 1854. Pathogenic V. cholerae produces a heat-sensitive enterotoxin that causes the characteristic cholera symptoms, including "rice water stool." The species comprises several somatic (O) antigen groups, including O-group-1, which is associated with classical and El Tor biotypes. V. cholerae O1 may have several serotypes, including Inaba, Ogawa, and Hikojima. V. cholerae non-O1 (referred to in older literature as nonagglutinable or NAG vibrios) also can cause gastrointestinal disease, though less severe than that caused by V. cholerae O1 (35). Non-O1 V. cholerae is found more readily in estuarine waters and seafood in the United States than is the O1 serogroup. Because this species can grow in media lacking sodium chloride, it is not considered a halophilic vibrio, although traces of sodium ion are required for growth. The FDA method for recovery of V. cholerae is qualitative; however, a most probable number (MPN) procedure can be used if necessary. Testing V. cholera O1 isolates for production of cholera toxin is recommended.

Table 1. Association of Vibrio spp. with different clinical syndromes[a,b]

	Clinical Syndrome				
Species	Gastroenteritis	Wound Infection	Ear Infection	Primary Septicemia	Secondary Septicemia
V. cholerae O1	+++	+			
V. cholerae non-O1	+++	++	+	+	+
V. mimicus	++		+		
V. fluvialis	++				
V. parahaemolyticus	+++	+	+		+
V. alginolyticus	(+)	++	++	+	
V. cincinnatiensis				+	
V. hollisae	++			+	
V. vulnificus	+	++		++	++
V. furnissii	(+)				
V. damsela		++			
V. metachnikovii	(+)			(+)	
V. carchariae		+			

[a] +++ = frequently reported, ++ = less common (6–100 reports); + = rare (1–5 reports); and (+) = association is unclear.

[b] Table taken from A.T. Pavia et al. (22).

Some diarrheal and otitis isolates, once thought to be atypical V. cholerae non-01 (sucrose-negative), are now recognized as a separate species, V. mimicus (5,25). Members of the species may produce cholera-like enterotoxins. V. mimicus can be identified by biochemical procedures used for the identification of V. cholerae.

V. parahaemolyticus is a halophilic bacterium found naturally in estuarine waters and animals. It was first described as the cause of gastroenteritis in Japan (9) and was first found in the United States by Baross and Liston (1) in the estuarine waters of Puget Sound. It has a worldwide distribution in estuarine and coastal environments and has been isolated from many species of fish, shellfish, and crustaceans. V. parahaemolyticus has been implicated in numerous outbreaks of seafood-borne gastroenteritis in the United States. Between 1971 and 1978, crab, oyster, shrimp, and lobster were implicated in 14 outbreaks, which may have resulted from the consumption of raw or insufficiently heated seafood or properly cooked seafood contaminated after cooking. The FDA method of enumeration uses an MPN format.

V. vulnificus is a halophilic bacterium found in the estuarine environment and is similar phenotypically to V. parahaemolyticus (21). The species was first described as "lactose-positive" because most strains ferment lactose and are o-nitrophenyl-beta-D-galactosidase (ONPG) positive. It causes foodborne and wound disease, either of which may progress to rapidly fatal septicemia, especially in individuals with liver disease (cirrhosis) or other underlying illnesses such as diabetes. Raw oysters are the major source of foodborne disease caused by V. vulnificus. The FDA method of enumeration uses an MPN series confirmed by biochemical testing or an immunological test, such as the ELISA, with monoclonal antibody to a species-specific intracellular antigen.

Other halophilic Vibrio spp., including V. fluvialis, V. hollisae, V. alginolyticus, V. furnissii, and V. metschnikovii, have been associated with gastroenteritis and are present in estuarine environments along with other pathogenic and nonpathogenic species of Vibrio. V. cincinnatiensis, V. damsela, and V. carchariae have not been associated with gastroenteritis, but are pathogenic to humans (Table 1). V. anguillarum, V. damsela, and V. carchariae are pathogenic to fish. Biochemical testing is required for taxonomic speciation.

NOTE: Although many of the same media are used for the enrichment and identification of various Vibrio spp., procedures have been optimized for detection of V. cholerae and for enumeration of V. parahaemolyticus and V. vulnificus. Vibrio species other than these may be encountered on thiosulfate-citrate-bile salts-sucrose (TCBS) agar and can be identified by the characteristics given in Tables 2-4 and in supplementary literature such as Bergey's Manual of Systematic Bacteriology (3). All of the cultural and serological tests should be performed in conjunction with known strains for comparative purposes.

V. hollisae does not grow readily on TCBS agar and a selective agar has not been developed. If V. hollisae is to be detected, a differential medium such as blood agar flooded with oxidase reagent after incubation (13) or mannitol-maltose agar (20) may be used.

A. Equipment and materials

1. Same as for Salmonella, Chapter 5
2. Water baths, 35-37°C and 42°C
3. Incubator, 39-40°C
4. Commercial bacterial identification strips (API 20E)
5. VET-RPLA TD20 kit (Oxoid) for cholera toxin
6. V-bottom (conical) microwell plates for VET-RPLA kit assay
7. Low protein-binding 0.2 µm filters (Millipore SLGV)
8. Micropipettors or micro-repeating pipettors
9. Tissue culture cluster plates, 96-well (CoStar, Cambridge, MA)
10. Flat-bottomed ELISA (EIA) plates, 96-well (CoStar or Immulon 2, Dynatech Laboratories, Alexandria, VA)
11. ELISA plate washer (optional)
12. ELISA (EIA) plate reader
13. Shaker incubator, 35-37°C
14. Sterile syringes, 1 ml
15. Centrifuge
16. Swiss Webster mice, 20 g
17. Isolator cages for mice
18. For gene probes, see Chapter 24
19. For tissue culture: cell culture flasks; cell culture 96-well plates; CO_2 incubator, 37°C; inverted phase-contrast microscope
20. 10,000 M.W. cutoff ultrafiltration membranes (PM10 or YM10; Amicon, Inc., Danvers, MA) and apparatus
21. Glass petri dishes for membrane ELISA
22. Nitrocellulose membranes, 82 mm (Bio-Rad Labs or Schleicher and Schuell)

B. Media and reagents

NOTE: Halophilic Vibrio spp. require added NaCl (2-3% final concentration). V. cholerae grow well in media with 0-3% NaCl. Add NaCl to media as specified in Appendix 3.

1. AKI broth (M7)
2. Alkaline peptone agar slants (M8)
3. Alkaline peptone salt (APS) broth (M9)
4. Alkaline peptone water (APW) (M10)
5. Anti-H (flagellar) sera-coated latex reagent for V. vulnificus agglutination, available from E.L. Elliot
6. Arginine glucose slant (AGS) (M16)
7. Bicarbonate buffer, 0.1 M, pH 9.6 (R4)
8. Blood agar (M20)
9. 1% Bovine serum albumin (BSA) in ELISA buffer (R6) and in PBS (R7)
10. Brain heart infusion (BHI) agar (M24)
11. Bromcresol purple broth (M26) supplemented individually with sucrose, lactose, cellobiose, arabinose, D-mannitol, or D-mannose
12. Cary-Blair transport medium (M31)
13. Casamino acids-yeast extract (CYE) broth (M32)
14. Chicken red blood cells, 2.5%, in physiological saline
15. Cholera enterotoxin (Becton-Dickinson Immunodiagnostics, Orangeburg, NY 10962; Sigma Chemical Company, St. Louis, MO 63178; or List Biological Laboratories, Inc., Campbell, CA 95008
16. Christensen's urea agar (M40)

17. Citric acid, 0.05 M, pH 4.0 (R13)
18. Coating solution for V. vulnificus EIA (R15)
19. Decarboxylase basal medium (M44), unsupplemented, and supplemented individually with arginine, lysine, or ornithine
20. ELISA buffer for cholera toxin assay (R22)
21. El Tor phage V (ATCC, Rockville, MD)
22. Enzyme immunoassay (EIA) wash solution (R21) for V. vulnificas
23. Fetal calf serum
24. Formalinized phosphate-buffered saline (R26)
25. Gelatin agar (GA) (M54) and gelatin salt (GS) agar (M55)
26. Goat antiserum to cholera toxin (CT) (List Biological Laboratories)
27. Gram stain dyes (R32)
28. Ham's F-10 medium with glutamine (M58), commercial preparation preferred
29. Heart infusion broth and agar (M60)
30. Hugh-Leifson glucose broth (M60)
31. 1 N hydrochloric acid (HCl) (R36)
32. Iron dextran, sterile, commercially available (Sigma; IMFERON, Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH)
33. Kligler iron agar (KIA) (M71)
34. Long-term preservation medium (M85)
35. Mannitol-maltose agar (M96)
36. Methyl red indicator (R44)
37. Mineral (paraffin) oil, sterile (R46)
38. Modified cellobiose-polymyxin B-colistin (mCPC) agar (M98)
39. Monoclonal antibody to V. vulnificus (contact E.L. Elliot)
40. Motility test medium, semisolid (M103)
41. MR-VP broth (M104)
42. Mueller-Hinton agar (M107)
43. Mukerjee phage IV
44. O/129 disks, 10 and 150 µg (R51)
45. OF medium, semisolid (M116), supplemented individually with glucose, sucrose, lactose, cellobiose, arabinose, D-mannitol, or D-mannose
46. ONPG test reagents (R53) or commercially available disks
47. Oxidase test reagent (R54)
48. Penicillin-streptomycin (Pen-strep) solution (M119)
49. Peroxidase-conjugated goat immunoglobulin G (anti-mouse; anti-rabbit)
50. Peroxidase substrate solution (ABTS) (R58)
51. Phosphate-buffered saline (PBS), pH 7.4 (R59)
52. Physiological saline solution, 0.85% (R63)
53. Polymyxin B antibiotic disks, 50 units (R64) or commercially prepared
54. Poly, Inaba, and Ogawa antisera for V. cholerae (Difco; Burroughs-Wellcome). Monoclonal antibody reagents are available from Denka Seiken Co., Tokyo, Japan, through Nichimen America, Inc., 1185 Avenue of the Americas, New York, NY 10036. For monoclonal antibody to V. cholerae O1 antigen, contact E.L. Elliot.
55. Rabbit antiserum to cholera toxin (contact E.L. Elliot)
56. Reagents for gene-probe testing, see Chapter 24)
57. Reference strains for V. cholerae classical and El Tor biotypes, serogroup O1; cytotoxin-producing strains of V. cholerae serogroup non-O1; reference strains of Kanagawa phenomenon-positive V. parahaemolyticus; cytotoxin-hemolysin-positive strains of V. vulnificus, and V. mimicus (contact E.L. Elliot)

58. Sera for O and K antigen testing of O (somatic) and K (capsular) antigens of V. parahaemolyticus (Denka Seiken Co., through Nichimen America, Inc.)
59. Sodium chloride dilution water, 2% and 3% (R71)
60. 1 N sodium hydroxide (NaOH) solution (R73)
61. Sheep red blood cells, 5%, in physiological saline
62. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (M147)
63. Triple sugar iron (TSI) agar (M149)
64. Tris-buffered saline, pH 7.5 (TBS) (R81); with 1% or 3% gelatin (R82); with 0.05% Tween 20 (TBS-Tween (R83)
65. Tris-EDTA-Triton X-100 (TET) buffer (R85)
66. Triton X-100 (R86)
67. Trypsin-EDTA solution, 1X (R87)
68. Tryptic soy broth (TSB) without dextrose, supplemented with 1% NaCl and 24% glycerol (M155) (total 1.5% NaCl), and with 2.5% NaCl (total 3% NaCl)
69. Trypticase (tryptic) soy agar (TSA) (M152) and broth (TSB) (M154)
70. 1% Tryptone, 1% NaCl (T_1N_1) agar (M163) and T_1N_2 agar
71. Tryptone broth (1%), unsupplemented (0% NaCl) (M164), or supplemented with 1, 3, 6, 8, or 10% NaCl (M161)
72. Voges-Proskauer test reagents (R89)
73. Wagatsuma agar (M178)
74. Y-1 adrenal cell growth medium (M180)
75. Y-1 mouse adrenal cells or Chinese hamster ovary (CHO) cells (ATCC)

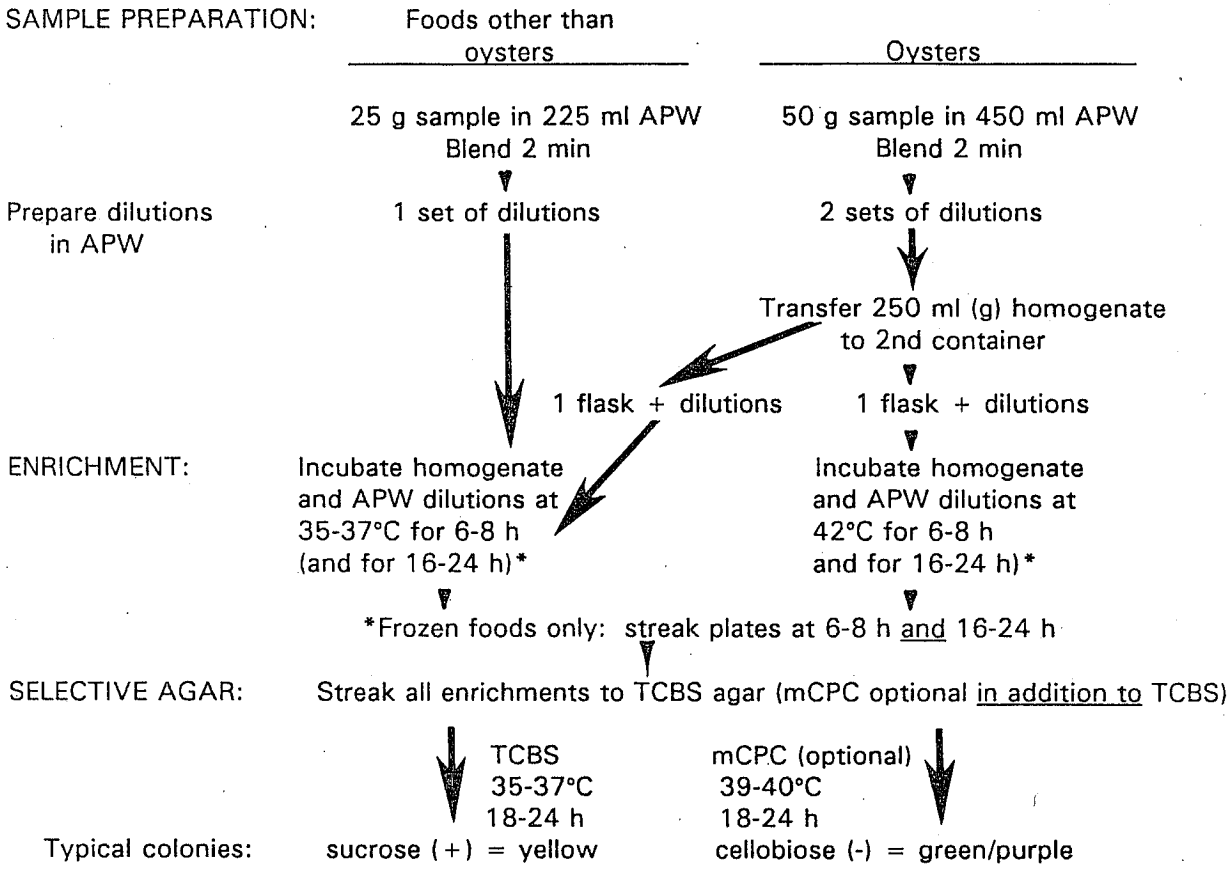
C. Procedure for enrichment and isolation of V. cholerae and V. mimicus from foods (Fig. 1). For stool, rectal swab, or vomitus specimens, see D-1, below, for transport and initial inoculation procedures.

1. Sample preparation. Aseptically weigh 25 g sample into 500 ml tared sterile blender jar or Stomacher. Cut large samples into smaller pieces before blending. Add 225 ml alkaline peptone water (APW) to jar and blend for 2 min at top speed. For oysters only (especially oysters freshly harvested from warm waters), prepare composite of 10-12 animals including shell liquor; blend to mix. Blend 50 g of this composite with 450 ml of APW. Pour 250 ml (g) into another sterile container.

   NOTE: Isolating specific Vibrio spp. from samples containing high concentrations of enteric bacteria may be difficult because of overgrowth by the enterics. For samples such as vegetables, estuarine waters, and other environmental samples expected to have high numbers of enterics, dilute the blended samples to a final 1:100 dilution and proceed as usual. For example, pour the blended sample [25 g blended with 225 ml alkaline peptone water (APW)] into a large flask or jar containing 2250 ml APW. If large sterile containers are not available, dilute the blended sample dilution 1:10 into smaller portions.

   For seafood samples, especially oysters, also prepare tenfold dilutions of the blended seafood sample in 9 (or 90) ml APW blanks (1:100 and 1:1000 dilutions) and proceed as usual. Prepare 2 sets of dilution tubes for oysters. Dilutions are made to decrease competition from other vibrios.

Figure 1. SCHEMATIC DIAGRAM FOR Vibrio cholerae ISOLATION METHOD



PURIFICATION:

Pick typical colonies to T_1N_1 Agar or TSA + 1.5% NaCl
(Optional: Inoculate GA + GS with same inoculum)

12-24 h, 35-37°C

PRELIMINARY ID TESTS:

MEDIUM	TYPICAL RXN OF V. cholerae
TSI	A/A--
KIA	K/A--
AGS	K/a--
T_1N_0 and T_1N_3	Growth (+)
GA and GS	Growth and gelatinase (+)
Hugh-Leifson glucose broth	Oxidative and fermentative
Oxidase test	+
Gram stain	Gram (-) rods (may be curved)

SEROLOGY: Group (polyvalent): V. cholerae O1 or non-O1
O1 subtype: Inaba, Ogawa, Hikojima

BIOCHEMICAL TESTS including BIOTYPE: See method

TOXIGENICITY: Tissue culture, ELISA, RPLA, or gene probe

Dilutions prepared for V. parahaemolyticus and V. vulnificus analyses may also be used. If sample is to be tested for all three Vibrio species (and others), use a sample large enough to inoculate all required media, and prepare the homogenate in APW or PBS, pH 7.2-7.5. For example, if sample is to be analyzed for V. cholerae, V. parahaemolyticus, and V. vulnificus, homogenize a 50 g sample with 450 ml APW. Place 250 ml (g) of APW homogenate in sterile container and follow the method for V. cholerae. (If PBS is used during homogenation, transfer 250 ml (g) of PBS homogenate to 2250 ml APW.) With the remainder, prepare dilutions in PBS, pH 7.2-7.5, inoculate MPN tubes of APW, and incubate tubes at 35-37°C. These tubes will serve as MPN enrichment tubes for V. parahaemolyticus and V. vulnificus, as well as V. cholerae in materials that may have high background microflora. From APW, inoculate selective plating media at 6-8 h for V. cholerae (if samples have not previously been frozen) and at 16 h for V. parahaemolyticus and V. vulnificus. For frozen foods tested for V. cholerae, incubating enrichment broths for 16-24 h may be necessary before inoculating selective agar. For oyster samples to be tested for the three Vibrio species, use a sample of at least 75 g since two 250 ml (g) test portions of APW homogenate are incubated for V. cholerae analyses (one at 35-37°C and one at 42°C).

2. Leave blended solutions in jars or pour into loosely stoppered sterile 500 ml Erlenmeyer flasks and incubate jars, flasks, and dilutions 6-8 h at 35-37°C. Incubation should not exceed 8 h.

   a. Exception. Incubate second jar or flask of oyster homogenate and one set of dilutions at 42°C for 6-8 h (6).

   b. Exception. Incubate frozen or otherwise processed food homogenate and dilutions 6-8 h, plate inocula to isolation agar (see 3, below), and reincubate enrichment broths for total incubation time of 18-24 h. Plate the 24 h enrichment broths to isolation agar.

3. After incubation, and without shaking flask, transfer 3-5 mm loopful of inoculum from pellicle (surface growth) onto at least one plate of selective plating medium: TCBS agar. (mCPC agar is optional; it may be used in addition to TCBS. Classical biotype V. cholerae is inhibited on mCPC agar by polymyxin B.) Incubate TCBS agar for 18-24 h at 35-37°C and mCPC agar for 18-24 h at 39-40°C.

4. Examine plates for colony characteristics described below. Carefully pick 3 or more suspect colonies from each plate, streak for isolation on T_1N_1, T_1N_2, or tryptic soy agar (2% total NaCl concentration), and incubate for 12-18 h at 35-37°C. Streaking for isolation on nonselective medium may be necessary to ensure colonial purity before biochemical testing. Gelatin agar (GA) and gelatin salt (GS) agar (see 5b, below) may also be inoculated with the same inoculum.

   a. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar. On TCBS agar, V. cholerae (El Tor and classical) are large, smooth, yellow (sucrose-positive), and slightly flattened colonies with opaque centers and translucent peripheries. NOTE: Vibrio spp. do not produce tiny, creamy yellow colonies on TCBS agar. Colonies of

closely related V. mimicus are green (sucrose-negative). Most other Vibrio spp. grow on TCBS agar and produce yellow or green colonies.

b. Modified cellobiose-polymyxin B-colistin (mCPC) agar (30). Colonies of V. cholerae E1 Tor are green-to-purple (cellobiose fermentation-negative). V. vulnificus produces flattened yellow colonies with opaque centers and translucent peripheries. Most other Vibrio spp. do not grow readily on CPC agar (19) or mCPC agar.

5. Distinguish suspect vibrios from non-vibrios

a. TSI, KIA, and arginine glucose slant (AGS). Inoculate individual colonies into TSI or KIA, and AGS media by stabbing butt and streaking slant. Incubate loosely stoppered or capped inoculated slants 18-24 h at 35-37°C. These media are recommended because the reactions permit early presumptive differentiation between most Vibrio spp., Aeromonas spp., Plesiomonas shigelloides, and other bacteria (Tables 2 and 3).

b. 1% Tryptone (tryptophane) broth (T_1N_0) and broth containing 3% NaCl (T_1N_3). Inoculate individual colonies into T_1N_0 and T_1N_3 broths and incubate 18-24 h at 35-37°C. Reincubate growth-negative tubes an additional 18-24 h. V. cholerae and V. mimicus will grow in T_1N_0 and T_1N_3. Some non-vibrio bacterial species producing reactions similar to those of V. cholerae in TSI and LIA media will not grow in T_1N_3. Most Vibrio spp., including some V. cholerae non-01, will grow in T_1N_3 only.

Alternatively, gelatin agar (GA) and gelatin agar with 3% NaCl (GS) can be used to screen isolates for salt tolerance (29). Divide plates into 8 sectors. Inoculate a straight line in the center of one sector of both GA and GS plates with each isolate. Incubate 18-24 h at 35°C. V. cholerae and V. mimicus will grow on both plates because they do not require salt. Halophilic Vibrio spp. will grow only on the GS plate. To read the gelatinase reaction, hold plate above a black surface. An opaque halo will be present around growth of gelatinase-positive organisms.

c. Oxidation-fermentation test. Inoculate 2 tubes of Hugh-Leifson glucose broth or OF glucose medium (semisolid) with growth from an isolated colony. Overlay medium in one tube with sterile mineral oil or liquid Vaspar (50% petrolatum, 50% paraffin) to depth of 1-2 cm and incubate 1-2 days or more at 35-37°C. Acid causes dye to change from purple to yellow in Hugh-Leifson broth, and from green to yellow in OF medium, semisolid. Vibrio spp. ferment glucose and produce acid from glucose oxidatively. Pseudomonas spp., commonly isolated from seafood by enrichment methods used for Vibrio spp., utilize glucose oxidatively only.

d. Oxidase test. Perform oxidase test on 18-24 h growth from TSA or other medium containing no fermentable carbohydrate such as GA or GS. An easy rapid method is to place a filter paper circle in a petri plate and moisten the entire filter paper with a few drops

Table 2. Reactions[a] of certain vibrios and related microorganisms in differential tube agar media

Microorganism	KIA		TSI		AGS	
	Slant	Butt	Slant	Butt	Slant	Butt
V. cholerae	K	A	A(K rare)	A	K	a
V. mimicus	K	A	K(A rare)	A	K	A
V. parahaemolyticus	K	A	K	A	K	A
V. alginolyticus	K	A	A	A	K	A
V. vulnificus	K or A	A	K(A rare)	A	K	A
A. hydrophila	K or A	A	K or A	A	K	K
P. shigelloides	K or A	A	K or A	A	ND	ND

[a]K, alkaline; A, acid; a, slightly acid; ND, not determined. None of the listed Vibrio spp. produce hydrogen sulfide gas in KIA, TSI, or AGS media, or gas from glucose in detectable quantities in KIA, TSI, or AGS media. Some Aeromonas spp. may produce gas from glucose in these media.

---

of oxidase reagent. With a sterile wooden applicator stick, toothpick, or platinum loop, pick growth from the plate and touch the moistened paper. Oxidase positive organisms will turn the paper dark purple or blue within a few seconds. Pathogenic Vibrio spp. are oxidase-positive (except for V. metschnikovii).

6. Identification and confirmation of V. cholerae O1, V. cholerae non-O1, and V. mimicus.

a. Read results of TSI, KIA, AGS, T_1N_0 and T_1N_3 or GA and GS, and oxidation-fermentation tests.

b. Perform Gram stain on 18-24 h broth or agar culture.

NOTE: Isolates to be carried through the remaining V. cholerae serological and biochemical tests are sucrose-positive (yellow) on TCBS agar [sucrose-negative (green) for V. mimicus)] or cellobiose negative (green-purple) on mCPC agar. They grow in T_1N_0 and T_1N_3 broths or on GA and GS plates; show characteristic reactions (see Table 2) in TSI, KIA, and AGS; are gelatinase and oxidase-positive; are Gram-negative rods or curved rods; and produce acid from glucose both oxidatively and fermentatively in Hugh-Leifson glucose broth or OF glucose medium, semisolid.

Table 3. Biochemical characteristics of several of the Vibrionaceae

	V. alginolyticus	*V. anguillarum*	*V. carchariae*	*V. cholerae*	*V. cincinnatiensis*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. harveyi*	*V. hollisae*	*V. metschnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*	*A. hydrophila*	*P. shigelloides*	*Photobacterium spp.*
TCBS agar	Y	Y	Y	Y	Y	G	Y	Y	Y/G	NG	Y	G	G	G	Y	G	NG/G
mCPC agar	NG	NG	nd	P	nd	NG	NG	NG	nd	NG	NG	NG	NG	Y	NG	NG	NG
AGS medium	KA	nd	nd	Ka	nd	nd	KK	KK	nd	Ka	KK	KA	KA	KA	KK	nd	nd
Growth in:																	
0% NaCl	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	-	+	V	+	+	+	+	+	-	+	+	+	-	V
8% NaCl	+	-	+	-	-	-	V	+	V	-	V	-	+	-	-	-	-
10% NaCl	+	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-
Growth at 42 C	+	-	nd	+	-	-	V	-	V	nd	V	+	+	+	V	+	-
Acid from:																	
Sucrose	+	+	+	+	+	-	+	+	V	-	+	-	-	-	V	-	V
D-Cellobiose	-	+	+	-	+	+	+	-	nd	-	-	-	V	+	+	-	-
Lactose	-	-	-	-	-	-	-	-	V	-	-	-	-	+	V	-	-
Arabinose	-	V	-	-	+	-	+	+	-	+	-	-	+	-	V	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	-	+
D-Mannitol	+	+	+	+	-	-	+	+	+	-	+	+	+	V	+	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V
ONPG	-	+	-	+	+	-	+	+	V	-	+	+	-	+	+	-	+
Voges-Proskauer	+	+	-	V	+	+	-	-	-	-	+	-	-	-	+	-	V
Arginine dihydrolase	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	+	+
Lysine decarboxylase	+	-	+	+	+	V	-	-	+	-	+	+	+	+	V	+	V
Ornithine decarboxylase	+	-	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-
Sensitivity to:																	
10 ug O/129	R	S	R	S	R	S	R	R	R	nd	S	S	R	S	R	S	V
150 ug O/129	S	S	S	S	S	S	S	S	S	nd	S	S	S	S	R	S	S
Gelatinase	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	-	-
Urease	-	-	+	-	-	+	-	-	V	-	-	-	V	-	-	-	-

Table 3 (continued)

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin; AGS, arginine-glucose slant; Y, yellow; G, green; P, purple; NG, no growth; nd, not determined; K, alkaline; A, acid; a, slightly acid; +, 80% or more of strains positive; -, 80% or more of strains negative (fewer than 20% of strains positive); V, variable reaction depending on species or strain; S, sensitive; R, resistant. Arginine glucose slant (AGS) reactions: slant, butt; all strains tested were hydrogen sulfide and gas negative. ONPG: o-nitro-beta-D-galactopyranoside hydrolysis by beta-galactosidase. Biochemical reactions from refs 4,7,10,11,18,21,23,24,33,34.

---

c. Serological agglutination test. Use diagnostic antisera of Group O1 and subgroup Inaba (factors AC) and Ogawa (factors AB) to serotype O1 antigen. Use 16-24 h cultures from TSA. Include positive and negative cultures and saline controls for each antiserum used. Follow directions included with antisera. Drops of 10 µl are sufficient for the test. Because antigens in different species may be related, biochemical tests must be completed before isolate is confirmed as V. cholerae O1 or non-O1.

NOTE: Monoclonal antibodies are available, but anti-B and anti-C cross-react with bacteria of other species (26). Use polyclonal sera and/or monoclonal antibodies to the A antigen of the O1 complex.

Cultures that agglutinate in group O1 antiserum and not in plain physiological saline are V. cholerae group O1 if biochemical reactions confirm the isolate as V. cholerae. Cultures that agglutinate in this group-specific antiserum may be subtyped with Inaba and Ogawa antibodies.

Notify Dr. Joseph Madden, FDA (telephone 202/245-1217), or E.L. Elliot, of V. cholerae O1 isolates. Continue with biochemical characterization and toxigenicity determination of the isolates.

Cultures that agglutinate in poly (group O1) antiserum and in both Inaba and Ogawa antisera have all 3 factors (A, B, and C) and are serotype Hikojima.

Cultures that agglutinate in poly antiserum but not in Inaba or Ogawa antisera cannot be typed using these antisera.

Cultures confirmed biochemically as V. cholerae that do not agglutinate in Group O1 antiserum are V. cholerae non-O1.

Cultures that agglutinate in Group O1 antiserum and in saline cannot be typed. However, using a richer growth medium, such as heart infusion (HI) agar or BHI agar, may eliminate this autoagglutination.

d. Biochemical reactions (Table 3). For specific directions for biochemical tests, including O/129 sensitivity and ONPG tests, see D-3, below. Formulations for all biochemical media should include at least 2% NaCl. API strips may be used in lieu of conventional media, with 2% NaCl as diluent. For V. cholerae, use physiological saline (0.85% NaCl) as diluent.

e. Determination of classical and El Tor biotypes. Two biotypes of V. cholerae serogroup O1 (classical and El Tor) may be distinguished by the following methods (see Table 4). Use more than one test to differentiate biotypes. The easiest methods are polymyxin B sensitivity, hemolysin test, and Voges-Proskauer test.

Bacteriophage susceptibility. This method is a modification of that described by Finkelstein and Mukerjee (8). Inoculate HI broth with strain to be tested and incubate at 35-37°C for 4 h. Swab surface of Mueller-Hinton agar plate with 4 h broth culture to obtain confluent bacterial growth. Let plates absorb inoculum, and superimpose 1 loopful of approprate test dilution of phage IV onto agar surface with 3 mm platinum loop. Observe plate after overnight incubation at 35-37°C. Classical biotype strains are usually sensitive to this bacteriophage and will lyse on plate where phage was placed (indicated by clear plaque). El Tor biotype strains are resistant to this bacteriophage and will not be lysed (indicated by confluent growth).

Use this same method to test for sensitivity to El Tor phage V.

Polymyxin B sensitivity. This procedure is a modification of technique described by Han and Khie (12). Swab surface of Mueller-Hinton agar plate with 4 h HI broth culture (35-37°C) to obtain confluent growth. Let plates absorb inoculum and place 50 unit polymyxin B antibiotic disk on medium surface. Invert plates and incubate for 18-24 h at 35-37°C. Classical biotype strains will demonstrate zone of inhibition around disk (10-15 mm diameter). El Tor biotype strains will grow to edge of disk or will be inhibited slightly (7-8 mm diameter). Alternatively, use TSA, GA, or GS agar in place of Mueller-Hinton agar.

Hemolysin test. Mix equal volumes (0.5 or 1 ml) of 24 h HI broth culture and 5% saline suspension of sheep red blood cells. Set up similar mixtures with portion of culture that has been heated for 30 min at 56°C. Use known hemolytic and nonhemolytic strains of V. cholerae as controls. Incubate mixtures for 2 h in 35-37°C water bath, then refrigerate overnight at 4-5°C. Examine tubes for hemolysis. Low speed centrifugation may aid in detection of cell lysis. Most El Tor strains will lyse red blood cells. Heated portion of culture should produce no hemolysis because hemolysin is thermolabile.

Table 4. Differentiation of biotypes of V. cholerae O1[a,b]

Test	Classical	El Tor
Sensitivity to El Tor phage V	-	+
Sensitivity to classical phage IV	+	-
Sensitivity to polymyxin B, 50 units	+	-
Hemolysis (sheep erythrocytes)	-	v
Hemagglutination (chicken erythrocytes)	-	+
Voges-Proskauer	-	+

[a]From Baumann and Schubert (3) and Madden et al. (18).

[b]+, Positive; -, negative; v, strains vary.

Classical biotypes of V. cholerae and some strains of biotype EI Tor will not lyse red blood cells. Alternatively, spot inoculum onto blood agar plates containing 5% sheep red blood cells, as for Kanagawa phenomenon (see below). Incubate at 35°C for 24 h and check for beta-hemolysis surrounding colonies.

Chicken red blood cell agglutination. Prepare thick, milky bacterial suspension in physiological saline from 18 to 24 h TSA culture. On clean glass slide, mix 1 loopful of washed chicken red blood cells (2.5% in physiological saline) with suspension of bacterial culture to be tested. Visible clumping of red blood cells indicates El Tor biotype. Classical strains usually do not agglutinate red blood cells. Perform positive and negative controls.

Voges-Proskauer (VP) test. Perform test in MR-VP broth after 18-24 h incubation at 22°C. El Tor biotype strains are usually positive; classical strains are negative.

f. Minimal characters for biochemical identification of V. cholerae. The following characteristics are presumptive of V. cholerae:

- Morphology: Gram-negative asporogenous rod or curved rod
- TSI appearance: Acid slant/acid butt, gas production-negative, H_2S-negative
- Hugh-Leifson test: Glucose fermentation- and oxidation-positive
- Cytochrome oxidase: Positive
- Arginine dihydrolase test: Negative
- Lysine decarboxylase test: Positive
- Voges-Proskauer test: El Tor biotype-positive, classical biotype-negative; V. mimicus-negative
- Growth at 42°C: Positive
- Halophilism test: 0% NaCl-positive; 3% NaCl-positive; 6% NaCl-usually negative. Some strains of V. cholerae non-O1 may not grow in 0% NaCl.

- Sucrose fermentation: Positive (negative for V. mimicus)
- ONPG test: Positive
- Arabinose fermentation: Negative
- O/129 sensitivity: Sensitive to 10 and 150 µg O/129

7. Detection of cholera enterotoxin (CT) and cytotoxin

Test isolate determined to be V. cholerae or V. mimicus biochemically and/or serologically for CT by direct or immunological test. Direct tests include the effect of toxin in vitro on Y-1 mouse adrenal cells or Chinese Hamster Ovary (CHO) cells, and the in vivo suckling mouse assay (2). Immunological methods include ELISAs and latex agglutination tests. DNA probes are available for CT-like enterotoxin gene sequences (see Chapter 24).

a. Y-1 Adrenal cell or Chinese Hamster Ovary (CHO) cell assay for enterotoxin (CT) and cytotoxin

Cell culture flasks. Using standard cell culture techniques, grow Y-1 or CHO cells on surface of 25 cm sq plastic cell culture flasks, using 5 ml Y-1 cell growth medium at 37°C in 5% CO_2 incubator. Replace medium after 48 and 96 h and observe appearance of cells, using inverted phase-contrast microscope. Test cells before they become totally confluent in flask. Before preparing fresh flasks or monolayers in wells of microwell plates, wash monolayer with 5 ml sterile 0.85% saline or PBS. Add 0.5 ml 1X trypsin-EDTA solution and incubate at 37°C for 15 min. Cells can be dislodged from surface by tapping flask against hand. To stop trypsin activity, add 4.5 ml Y-1 growth medium (total volume in flask, 5 ml), wash cells from surface of flask, and transfer cell suspension to sterile tubes. Let large clumps of cells settle for 2 min. To prepare new flask of cells, add 1 ml cell suspension and 4 ml Y-1 growth medium to new 25 cm sq tissue culture flask.

Preparation of microwell plates. Add 15 ml Y-1 growth medium to 5 ml cell suspension prepared above. Transfer 0.2 ml of this diluted cell suspension to each of 96 wells. Incubate plates in CO_2 incubator at 37°C. When cell monolayers are confluent (usually within 3 days), plate is ready for use.

Preparation and concentration of test filtrates. Use AKI medium to enhance CT production by V. cholerae El Tor (15). Inoculate 15 ml AKI tubes and incubate 4 h without shaking at 35-37°C. Then transfer entire volume to 250 ml flask and incubate 16 h with shaking (200 rpm) at 35-37°C. Centrifuge culture at 9000 x g for 30 min in refrigerated centrifuge. Discard cells. Filter-sterilize supernatant, using 0.22 µm low protein-binding membrane, before testing. If large volumes of supernatant are collected, concentrate by ultrafiltration through 10,000 m.w. exclusion membrane, such as Amicon PM10 or YM10.

Optional: Inoculate CYE broth with isolate and incubate for 18-24 h at 35-37°C. Transfer 0.1-0.2 ml of this culture into 125 ml

flask containing 25 ml CYE broth. Incubate with shaking (100-200 rpm) for 24 h at 35°C.

Prepare supernatant as described above.

Assay. Remove old Y-1 medium and drain microwell plate by inverting on sterile towel. Add 0.1 ml fresh medium per well. Add 0.05 ml of each filter-sterilized bacterial culture supernatant to microtiter plate wells. For CT assay, use 0.05 ml of cholera enterotoxin (5 ng/ml) for positive control. For cytotoxin assay, use 0.05 ml of V. cholerae 2194C culture supernatant for positive control. Use sterile CYE medium for negative control. For second negative control well, boil culture supernatant from V. cholerae 2194C for 5 min. Incubate overnight at 35-37°C in CO_2 incubator.

Interpretation. Examine wells at 100 or 200X magnification, using inverted stage phase microscope. Compare test wells to positive control wells. For CT assay, a positive well contains more than 10% rounded Y-1 cells. For cytotoxin assay, a positive well contains 50% or more dead, lysed, and/or detached cells. El Tor strains are typically hemolytic (cytotoxic). Consequently, CT cannot be detected unless its concentration exceeds that of cytotoxin, and the sample is titered (various dilutions are used in assay).

Chinese hamster ovary (CHO) cell alternative. CHO cells may be used rather than Y-1 cells. CHO cells elongate when exposed to CT.

b. Immunological detection of cholera enterotoxin (CT): microwell plate and membrane ELISAs.

Screen suspect colonies for production of these toxins with appropriate dilutions of specific antibodies against CT, using membrane ELISA, a 96-well microELISA, or reversed-passive-latex agglutination (RPLA) assay kit in 96-well format. As with all immunological tests, when suppliers or stocks of antibody or conjugate change, the new reagents must be titered to determine optimum test dilutions.

Grow isolates in CYE medium with shaking (100-200 rpm) at 35-37°C, as described in a-3, above, for the Y-1 assay (AKI medium optional). Centrifuge at 9000 x g for 20 min in refrigerated centrifuge. Decant supernatant into sterile flask and discard cells. To obtain clear supernatants, filter through low protein-binding membrane. Supernatants need not be sterilized and may be frozen at -20°C before use.

To detect low amounts of toxin in culture supernatant, concentrate toxin using method of Yamamoto et al. (35).

1) Micro ELISA

Coat each well of a flat-bottomed, 96-well ELISA plate with 50 µl of appropriate dilution (e.g., 1:1000) of goat anti-cholera toxin diluted in 0.1 M bicarbonate buffer, pH 9.6. Cover and place at 4°C overnight. Wash plates 3 times with ELISA buffer. To reduce nonspecific binding, block wells with 200 µl 1% bovine serum albumin (BSA) in ELISA buffer for 30 min at 25°C. Wash 3 times with ELISA buffer.

Add 100 µl test substance to each well. Run positive controls (enterotoxin-producing bacterial strains) and negative controls (uninoculated growth medium). Cover and incubate plates for 1 h at 37°C. Wash plates 3 times with ELISA buffer.

Add 100 µl of appropriately diluted (in 1% BSA in ELISA buffer) rabbit anti-CT to each well. Cover and incubate plates at 37°C for 1 h. Wash plates 3 times with ELISA buffer. NOTE: Titrate rabbit anti-CT before use. A 1:500 dilution has been used successfully with some preparations.

Add 100 µl of appropriately diluted goat anti-rabbit peroxidase conjugate (e.g., 1:1000 in 1% BSA in ELISA buffer) solution to each well. Cover plates and incubate at 37°C for 1 h. Wash plates 3 times with ELISA buffer.

Add 100 µl of ABTS solution to each well. Cover and incubate plates at 37°C for 10-30 min. If necessary, reincubate plates at 37°C to obtain darker reactions.

Results. Read optical density of each well at 410 nm on spectrophotometer [ELISA (EIA) reader]. Culture supernatants yielding optical density 0.1 unit greater than background are positive for production of either CT or cytotoxin.

2) Reversed-passive-latex agglutination (RPLA) assay (Oxoid VET-RPLA TD20). This assay tests supernatants after simple centrifugation or filtration of 24 h, 35-37°C AKI or CYE medium culture. See kit for instruction on assay set-up following supernatant preparation.

3) Membrane ELISA

Fill large petri dish with 50 ml TBS. Handle nitrocellulose membrane with forceps and gloves. Mark membrane with pencil for future orientation. Place membrane at angle into TBS buffer to wet thoroughly. Remove after 10 min and place on filter paper for 5 min. Displace 2 µl of each culture supernatant, including positive and negative control cultures and uninoculated medium, to tip of micropipet and touch to membrane surface. Place each sample 6-8 mm apart. To avoid increasing spot size, let membrane dry completely before applying additional 2 µl aliquots to spots. Let membrane dry completely for 5 min after application of last sample. About 75 cultures can be analyzed on a single

membrane 9 cm in diameter. Immerse membrane in 50 ml TBS-3% gelatin solution for 1 h. Agitate solution intermittently or place on rotary shaker.

Remove membrane from TBS-3% gelatin and transfer to 50 ml solution of rabbit anti-CT antiserum diluted 1:100 (or other appropriate dilution) in TBS-1% gelatin. Incubate 2-3 h at 25°C with gentle agitation. Briefly rinse membrane in 50 ml double distilled water. Wash membrane 5 min with 50 ml TBS-Tween-20. Repeat washing procedure 3 more times.

Transfer membrane from wash solution to 50 ml goat antirabbit IgG-peroxidase conjugate diluted 1:3000 (or other appropriate dilution) with TBS-1% gelatin. Incubate at 25°C for 2 h with gentle agitation. Remove and wash membrane as in b, above.

Prepare ABTS peroxidase color development solution immediately before use. Transfer membrane from wash solution into color development solution. CT-positive spots will appear as purple dots within 5 min. Avoid prolonged color development beyond 15-30 min. If precipitate forms in color development solution, prepare fresh solution and use immediately. Immerse membrane in distilled water to stop color development.

Interpretation of data. Spots containing 1 ng or greater concentration of CT become visible as purple dots within 2-5 min. Negative control cultures may give a very faint spot if color development proceeds beyond 5 min. High titer sera should be diluted (1:200 or 1:400) to minimize background color changes that may be observed with CT-negative control cultures.

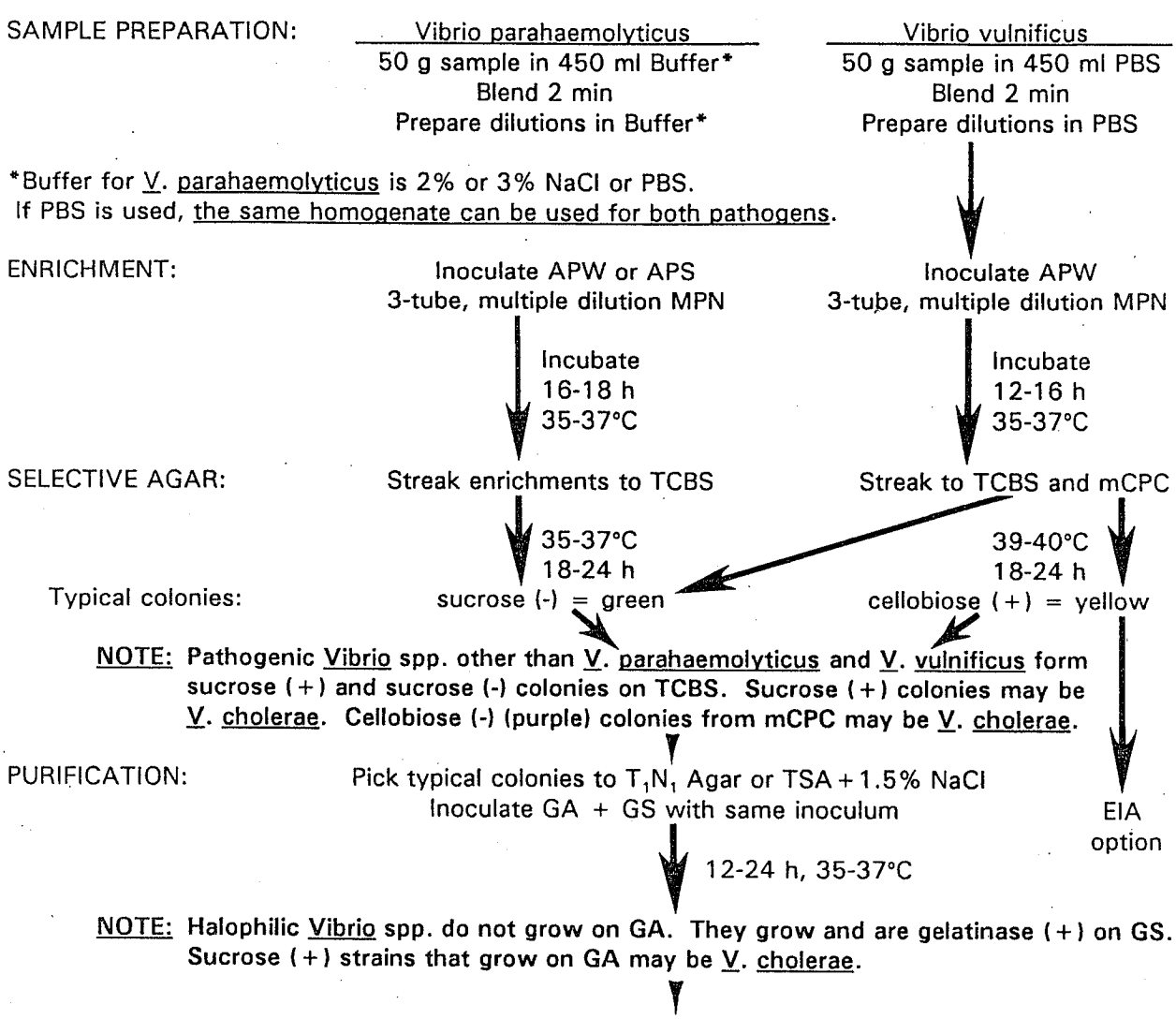
8. Report. The following should appear in the final report: identification of isolate based on biochemical test results; serological results (O1, Non-O1, Inaba, Ogawa, Hikojima); biotype of *V. cholerae* O1 (El Tor or classical); and toxigenicity results (toxigenic or nontoxigenic, and cytotoxic or noncytotoxic if tissue culture was used). Notify Dr. Joseph Madden, FDA (telephone 202/245-1217), or E.L. Elliot, of *V. cholerae* O1 isolates.

D. Procedure for enrichment, isolation, and enumeration of *V. parahaemolyticus*, *V. vulnificus*, and other halophilic *Vibrio* spp. (see Fig. 2)

1. Stool sample. The importance of obtaining stools, rectal swabs, or vomitus specimens in outbreaks cannot be overemphasized. Obtain specimens at the earliest opportunity because the carrier state is short-lived. Transport specimens in Cary-Blair medium, or inoculate immediately into APW enrichment broth, or streak onto TCBS and mCPC agar plates, depending on transit time. Incubate TCBS agar at 35°C and mCPC agar at 39-40°C for 18-24 h.

If transit time will be longer than 8 h, place stool specimen in Cary-Blair transport medium. In the laboratory, streak loopful onto TCBS and mCPC agars.

Figure 2. SCHEMATIC DIAGRAM FOR Vibrio parahaemolyticus and Vibrio vulnificus METHODS



PRELIMINARY BIOCHEMICAL TESTS:
(For other tests, see Chapter and Tables 2 and 3)

TEST	TYPICAL REACTIONS OF V. parahaemolyticus	V. vulnificus
Oxidase	+	+
Motility	motile	motile
AGS	K/A--	K/A--
TSI	K/A--	K(A rare)/A--
O/129 (10 ug)	Resistant	Sensitive
O/129 (150 ug)	Sensitive	Sensitive
ONPG	-	+
Gram stain	Gram (-) rods	Gram (-) rods

SEROLOGY:	O and K, optional	EIA or Latex co-agglutination
PATHOGENICITY TESTS:	Kanagawa phenomenon (may correlate with pathogenicity)	Iron-loaded mouse bioassay

If transit time is 8 h or less, place stool specimen in APW. After 12-16 h incubation at 35-37°C, streak enrichment broth on both TCBS and mCPC agars. Incubation times longer than 16 h result in progressively lowered bacterial viability.

Sample rectal swab specimen (preferably with polyester fiber-tipped swab), contained in 7 ml Cary-Blair transport medium or APW, by streaking onto both TCBS and mCPC agars.

Examine plates and proceed to biochemical identification.

2. Food sample

a. Sample composition

Fish: surface tissues, gut, or gills

Shellfish: entire interior contents of animal; pool 10-12 animals, homogenize, and remove 50 g from composite for test sample

Crustaceans: entire animal, if possible, or central portion of animal, including gills and gut

b. Test sample preparation

V. parahaemolyticus. Aseptically prepare 1:10 dilution of 50 g seafood in 450 ml 2 or 3% NaCl dilution water or PBS, pH 7.2-7.5, in sterile, tared blender jar. Blend 2 min at high speed. Prepare tenfold dilutions in 2 or 3% NaCl dilution water or PBS, pH 7.2-7.5. Inoculate 3-tube, multiple dilution, alkaline peptone water (APW) or alkaline peptone salt (APS) broth MPN series (i.e., add 1 ml portions of each 1:10 and higher dilution to sets of 3 tubes containing 10 ml APW or APS). Incubate tubes 16-18 h at 35-37°C. NOTE: Inoculations of MPN tubes must be completed within 15-20 min of dilution preparation.

V. vulnificus. Prepare APW original and 10-fold dilutions in PBS, pH 7.2-7.5. Inoculate MPN series as described for V. parahaemolyticus and incubate at 35-37°C for 12-16 h.

NOTE: If enrichment is for both V. parahaemolyticus and V. vulnificus, use PBS, pH 7.2-75, buffer and APW enrichment broth.

c. After incubation do not shake tubes. For all dilutions containing a turbid tube, and at least one dilution higher, streak TCBS agar with 1 loopful from top 1 cm of each enrichment broth. For isolation of V. vulnificus also streak on mCPC (29) agar. Also streak APW broth cultures of verified V. parahaemolyticus and V. vulnificus strains on TCBS and mCPC agar plates as controls for subsequent tests. Incubate TCBS at 35-37°C and mCPC at 39-40°C for 18-24 h. Optional: Use blood agar or mannitol-maltose agar, incubated at 35-37°C for 18-24 h, to detect V. hollisae.

d. Examine TCBS and mCPC agars for typical V. parahaemolyticus and V. vulnificus colonies. Pick 3 or more typical or suspicious colonies from each medium, inoculate sectors of GA and GS plates and streak T_1N_2 agar or TSA + 1.5% NaCl (final 2% NaCl concentration) for isolation. Incubate at 35-37°C for 18-14 h. AGS or other screening media may be inoculated at this time.

TCBS agar. On TCBS agar, V. parahaemolyticus, V vulnificus, V. mimicus, and V. harveyi are round, 2-3 mm diameter, green or blue-green colonies. V. alginolyticus, V. fluvialis, V. cholerae, V. metschnikovii, and some V. vulnificus colonies are larger and yellow (acid from sucrose fermentation).

mCPC agar. On mCPC agar, V. vulnificus colonies are flat and yellow (acid from cellobiose fermentation) with opaque centers and translucent peripheries, about 2 mm in diameter. This is a presumptive identification of V. vulnificus. Non-cellobiose fermenters, such as V. cholerae El Tor, appear as purple or green, raised colonies. V. parahaemolyticus rarely grows on mCPC. Other species of Vibrio do not grow readily on mCPC agar. Pseudomonads produce purple or green colonies and are frequently observed at low dilutions of sample.

For rapid confirmed identification of V. vulnificus, transfer colonies from mCPC agar to APW for the monoclonal antibody-EIA (30) (see F, Serology, below). Use isolates confirmed by EIA to compute MPN of V. vulnificus in sample.

A rapid agglutination test using antiflagellar serum may also be used to presumptively identify V. vulnificus (see F, Serology, below).

A specific gene probe method, available for detection of cytotoxin-hemolysin gene of V. vulnificus, may be used as an additional presumptive identification procedure (see Chapter 24).

Blood agar. Flood 18-24 h plate with oxidase reagent and pick oxidase-positive colonies (13). Because V. hollisae does not grow on TCBS or mCPC agars, this nonselective method may isolate the organism. However, overgrowth by other bacteria may be a problem.

Mannitol-maltose agar. On this nonselective medium, V. hollisae colonies are round, shiny, and purple (non-mannitol, non-maltose fermenting), whereas other Vibrio spp. are yellow (acid from mannitol and/or maltose fermentation) (20). Overgrowth by other bacteria may be a problem.

Enumeration. When suspect colonies are identified biochemically or serologically with EPA, apply MPN tables (Appendix 2) for final enumeration of species.

3. Procedure for biochemical identification

a. Read GS and GA plates. Halophilic Vibrio spp., will grow only on GS plates. Most Vibrio spp. are gelatinase-positive and will form an opaque halo around growth.

b. Oxidase test. Use growth from GS plate (or other medium with no fermentable carbohydrate) for oxidase test. Place 2 or 3 drops of oxidase test reagent on bacterial growth, or transfer small amount of growth with sterile toothpick or platinum loop to filter paper moistened with oxidase reagent. (Do not use nickel chromium loops.) Dark blue color should develop rapidly (within 2 min) for positive reaction. V. metschnikovii is the only oxidase-negative, pathogenic, halophilic Vibrio spp.

c. From isolated colonies, inoculate motility test medium, AGS, TSI, TSB, TSA slant, and TSA plate for O/129 sensitivity test (all with final 2% NaCl concentration) and incubate 18-24 h at 35-37°C. Use various tests in Tables 2 and 3 for identification.

NOTE: Before proceeding, make sure culture does not grow on GA, is gelatinase-positive, and is pure. Vibrio spp. cultures often have 2 colony morphologies, which may or may not be stable.

Motility test medium. Stab inoculum in center and to 2/3 the depth of motility test medium. Diffuse circular bacterial growth from line of stab is a positive test. V. vulnificus, V. parahaemolyticus, and related Vibrio spp. are motile. After 24 h, tightly cap tube and store at 20-25°C to preserve culture.

Arginine-glucose slant. Streak slant and stab butt of AGS, modified from Kaper et al. (16). Vibrio spp. do not produce H_2S or gas. Typical reactions of V. parahaemolyticus and V. vulnificus are alkaline (purple) slant and acidic butt (yellow) (Tables 2 and 3).

Triple sugar iron. Streak slant and stab butt of TSI agar. Vibrio spp. produce acidic butt (yellow) and do not produce gas or H_2S. V. parahaemolyticus produces alkaline slant (red). V. vulnificus usually produces an alkaline slant (red) (Table 2). Use this or other medium containing lactose as source of inoculum for ONPG test.

Inoculate tubes of TSA and TSB or motility test medium as source of inoculum for further testing.

O/129 vibriostat sensitivity. Use the disk diffusion method described above for polymyxin B sensitivity of V. cholerae O1 (c-6, e-2) or place disks on densely streaked area of an isolation plate (TSA with 2% NaCl final concentration). Use disks containing 10 and 150 µg of vibriostat O/129. Vibrio spp. are sensitive to 150 µg of O/129, but some are resistant to 10 µg of O/129. See Table 3 for differentiation based on sensitivity to 10 µg of O/129. Disks are commercially available or can be prepared in the lab. Alternatively, use TSA agar containing 10 or 150 µg of O/129 per ml.

d. Continue identification tests after finding typical reactions in media inoculated previously (Tables 2 and 3). Compute MPN of V. parahaemolyticus (see Appendix 2), based on number of tubes containing V. parahaemolyticus.

ONPG test. Perform ONPG test using portion of culture from TSI or other medium containing lactose. Use conventional tube test (preferred) in fume hood, or commercially available discs. Strip tests for ONPG are sometimes unreliable for Vibrio spp. V. vulnificus is ONPG-positive; V. parahaemolyticus is ONPG-negative.

Rapid test strips. Use multiwell (e.g., API 20E) strips as alternative to conventional tube format for biochemical tests. However, some Vibrio spp. will not grow in commercial test strip media when physiological saline (0.85% NaCl) is used as diluent. Use 2% NaCl as diluent, since most halophilic Vibrio spp. require higher concentration of NaCl (17). Compare reactions to those in Tables 2 and 3. If commercial test strips do not allow identification, continue with conventional tests.

Hugh-Leifson glucose broth or OF glucose medium, semisolid.

Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase. Inoculate 1 tube of each of the 3 media containing amino acid and 1 tube lacking amino acid. (The arginine reaction can also be read from the AGS tube; acid butt (yellow) from glucose fermentation means isolate is negative for arginine dihydrolase.) Overlay each tube with sterile mineral oil 1-2 cm thick, and incubate 4 days at 35-37°C. Examine tubes every day. Alkaline pH resulting from decarboxylation of amino acids turns medium purple (positive). Yellow color results from acid production from glucose fermentation (negative). Control tubes containing no amino acid should be yellow. Most V. parahaemolyticus and V. vulnificus strains are arginine dihydrolase-negative, lysine decarboxylase-positive, and ornithine decarboxylase-positive. Some V. vulnificus and V. parahaemolyticus are ornithine decarboxylase-negative. Rare strains of V. vulnificus are lysine decarboxylase-negative.

Salt tolerance. From TSB culture, inoculate 1 tube each of 1% tryptone broth containing 0, 1, 3, 6, 8, or 10% NaCl (T_1N_0, T_1N_1, T_1N_3, T_1N_6, T_1N_8, or T_1N_{10}), and incubate 18-24 h at 35-37°C. Consider only profuse growth as positive. Halophilic Vibrio spp. do not grow in broth containing 0% NaCl, but all Vibrio spp. grow in broth containing 3% NaCl. Various species have different salt tolerances that can be used for identification (Table 3).

Growth at 42°C. Inoculate prewarmed tube of TSB containing 2% NaCl with small loopful of 24 h TSB-2% NaCl culture. Incubate in 42°C water bath for 24 h. Consider only profuse growth as positive. V. cholerae, V. parahaemolyticus, V. alginolyticus, and V. vulnificus grow at 42°C.

Voges-Proskauer (VP) test. Inoculate MR-VP broth with growth from TSA slant and incubate 2 days at 35-37°C. Perform VP test. V. parahaemolyticus, V. vulnificus, and V. fluvialis are VP-negative.

Carbohydrate fermentation. From growth on TSA slant, inoculate 1 tube each of bromcresol purple or OF medium, semisolid, broth containing sucrose, lactose, D-mannitol, mannose, arabinose, or

cellobiose. Overlay medium with sterile mineral oil and incubate at 35-37°C for 4-5 days. Acidic fermentation turns medium yellow. Check tubes every 24 h and compare reactions to those in Table 3. Occasional strains of V. vulnificus are mannitol-negative.

Urea hydrolysis. Test presumptive V. parahaemolyticus for urea hydrolysis by inoculating Christensen's urea agar tubes or plates and incubating at 35-37°C for 18 h. V. parahaemolyticus strains vary in ability to hydrolyze urea. Urea hydrolysis may be correlated with certain somatic (O-antigen) groups.

NOTE: Urease-positive strains give API codes not found in the ID book. Call API for confirmation of strain.

Culture preservation. Inoculate semisolid, long-term preservation medium or motility test medium by stabbing deeply into agar. Incubate 24 h at 35-37°C. Tighten caps after 24 h to prevent dehydration. Alternatively, add layer of sterile mineral oil to 24 h cultures in motility test medium. Store cultures at room temperature after initial growth. Do not refrigerate. For long-term preservation, place 1 ml of 6-12 h TSB-2% NaCl culture and 0.1 ml sterile glycerol into sterile cryotubes. Freeze immediately at -70°C or in liquid nitrogen.

Kanagawa phenomenon. The Kanagawa reaction demonstrates the presence of a specific thermostable direct hemolysin (TDH) on Wagatsuma agar. A positive reaction correlates closely with pathogenicity of V. parahaemolyticus isolates. Strains recovered from seafood are usually Kanagawa-negative.

Some clinical isolates of V. parahaemolyticus produce related hemolysins but not TDH. Two other hemolysins, having sequence homology with TDH but exhibiting no hemolysis on Wagatsuma agar, were recently identified and purified (14).

Fresh human or rabbit red blood cells (within 24 h of draw) are necessary for preparation of Wagatsuma agar.

Spot droplet from 18 h TSB-3% NaCl culture on duplicate plates of well-dried Wagatsuma agar. Spot several cultures including verified positive and negative controls in circular pattern on plate. Incubate at 35-37°C and observe results in 24 h.

A positive test is zone of beta-hemolysis, i.e., sharply defined zone of transparent clearing of red blood cells around colony, without multiple concentric rings or greening.

Measure zone of hemolysis from edge of colony to outer edge of zone. Isolates that produce clear zone of hemolysis 3 mm or larger are considered Kanagawa phenomenon-positive and are presumed to be pathogenic. Isolates that produce clear zones of hemolysis of less than 3 mm may be weakly pathogenic and should be tested in rabbit ileal loop assay (32). No observation of plate beyond 24 h is valid.

A gene probe method for detecting TDH of V. parahaemolyticus is available. V. hollisae is positive for TDH by gene probe, but its hemolysin cannot be detected on Wagatsuma agar.

Pathogenicity test for V. vulnificus. The iron-loaded mouse bioassay differentiates virulent from avirulent isolates of V. vulnificus.

When selecting suspect colonies for pathogenicity testing, choose opaque colonies if both opaque and transparent colonies are present on TSA plates at 35°C (28). Grow isolates in 250 ml flasks containing 50 ml HI broth supplemented with 1% NaCl at 35-37°C on 250 rpm shaker until early stationary phase (8-12 h).

Approximately 2 h before inoculating mice, inject twenty-five 20 g mice with 250 µg of iron dextran/g body weight by intramuscular or intraperitoneal route. Harvest cells from 10 ml of culture by centrifugation (9000 x g for 20 min at 4-5°C). Wash twice with 10 ml sterile PBS, pH 7.2, and resuspend cells in 10 ml sterile PBS. Determine viable count by standard spread-plate or pour-plate methods using BHI agar containing 2% NaCl. Viable count should be about 10^9/ml.

Inoculate groups of 5 mice with approximately 1000, 100, 10, and 1 CFU/mouse in 0.5 ml volumes intraperitoneally. Inoculate 5 control mice with sterile PBS. Observe mice for 48 h and count number of deaths in each group. Pathogenicity endpoint is usually less than 100 CFU/20 g mouse. Deaths caused by 1000 or fewer V. vulnificus CFU with no deaths of control mice, indicates virulence. Avirulent strains have not been reported to kill iron-loaded mice (20 g) at concentrations of less than 10^6 CFU mouse.

E. Characteristics for biochemical identification of V. parahaemolyticus and V. vulnificus

The following characteristics are presumptive of V. parahaemolyticus or V. vulnificus:

- Morphology: Gram-negative asporogenous rod
- TSI appearance: V. parahaemolyticus, alkaline slant/acid butt, gas production-negative, H_2S-negative; V. vulnificus, alkaline slant (rarely acidic)/acid butt, gas production-negative, H_2S-negative
- Hugh-Leifson test: Glucose oxidation and fermentation-positive
- Cytochrome oxidase: Positive
- Arginine dihydrolase test: Negative
- Lysine decarboxylase test: Positive (rare V. vulnificus are lysine decarboxylase-negative)
- Voges-Proskauer test: Negative
- Growth at 42°C: Positive
- Halophilism test: V. parahaemolyticus: 0% NaCl-negative; 3, 6, and 8% NaCl-positive; 10% NaCl-negative or poor. V. vulnificus: 0% NaCl-negative; 3, 6% NaCl-positive; 8% NaCl-negative
- Sucrose fermentation: Negative (rare V. vulnificus are positive)
- ONPG test: V. parahaemolyticus, negative; V. vulnificus, positive

- Arabinose fermentation: V. parahaemolyticus, usually positive (variable); V. vulnificus, negative
- Sensitivity to O/129: V. parahaemolyticus: sensitive to 150 µg, resistant to 10 µg; V. vulnificus: sensitive to 10 and 150 µg.

F. Serology

1. Serological identification of V. parahaemolyticus. Determination of somatic (O) and capsular (K) serotypes of V. parahaemolyticus is not required for identification. Serotyping antisera are expensive and few, if any, FDA labs have the complete set. Contact E.L. Elliot for possible referral of isolates. For those who wish to determine the O and K antigens (Table 5), the following protocol is offered.

   a. Inoculate 2 slants of TSA-2% NaCl; incubate at 35-37°C for 18-24 h.

   b. Somatic (O) antigen

      Preparation. Wash growth from one TSA-2% NaCl slant with solution containing 2% NaCl and 5% glycerol; transfer to autoclavable centrifuge tube. Autoclave suspension at 121°C for 1 h. Centrifuge suspension at 4000 rpm for 15 min. Resuspend the packed cells in 2% NaCl. A heavy suspension is best for this slide agglutination test.

      Determination. With wax pencil, divide microscope slide into 12 equal compartments. Place small drop of heavy suspension into each compartment. Add 1 drop of the 11 O-group antisera to separate compartments. Add 1 drop of 2% NaCl to 12th compartment (autoagglutination control). Tilt slide gently to mix all components, and rock slide back and forth for 1 min. Positive agglutination may be read immediately.

      If no agglutination occurs with any of the 11 O antisera, autoclave the suspension at 121°C again for 1 h and retest. If agglutination is still negative, the O antigens of the culture are unknown.

   c. Capsular (K) antigen

      Preparation. Capsular (K) antigen. Wash growth from one TSA-2% NaCl slant with 2% NaCl solution to make a smooth heavy suspension of cells.

      Determination. Test first with pooled K antisera (I-IX), and then with each of the monovalent K antisera within the pool showing agglutination. (Each pool consists of 8-10 flagellar agglutinins.)

      On slide, mark off appropriate number of compartments plus control compartment. Place small drop of heavy cell suspension and add 1 drop of appropriate K antiserum to individual compartments. Add 1 drop of 2% NaCl to autoagglutination control. Tilt slide gently to mix components, and rock slide back and forth for 1 min. Positive agglutination may be read immediately.

Table 5. Antigenic scheme of V. parahaemolyticus[a]

O Group	K Antigen
1	1, 25, 26, 32, 38, 41, 56, 58, 64, 69
2	3, 28
3	4, 5, 6, 7, 29, 30, 31, 33, 37, 43, 45, 48, 54, 57, 58, 59, 65
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67
5	15, 17, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 39, 70
9	23, 44
10	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61

[a]From R. M. Twedt (31), personal communication from R. Sakazaki in 1986.

2. V. vulnificus EIA (30). Use EIA specific for intracellular antigen to confirm identity of V. vulnificus isolates directly from mCPC agar (yellow translucent colonies with opaque centers).

Prepare log phase cultures. Transfer 2 typical V. vulnificus colonies from each inoculated plate and confirmed culture of V. vulnificus, using sterile wooden sticks, tooth picks, or inoculating loop, to individual wells of 96-well plate (tissue culture cluster plate) containing 100 µl APW per well. Incubate 3-4 h, or until turbid, at 35-37°C.

Coat enzyme immunoassay (EIA) plates. After incubating microtiter plates, transfer 25 µl of each APW culture to 2 replicate wells of 96-well EIA plate (one is test well and one is negative control). Add 25 µl EIA coating solution (0.02% Triton X-100) to each well. Place EIA plates in dry 35°C incubator overnight to evaporate samples in wells.

Optional: To store isolates after transfer to EIA plates, add equal volume sterile TSB supplemented with 1% NaCl and 24% glycerol to each well of tissue culture plate. Isolates can be stored indefinitely at -70°C.

Block binding sites. Remove dried EIA plates from incubator. To reduce nonspecific binding of reagents, add 200 µl of 1% BSA in PBS to each well. Incubate at room temperature for 1 h.

Discard BSA. Remove BSA solution by firmly slapping plates onto countertop covered with absorbent towels.

Add monoclonal antibody. Prepare diluted (e.g., 1:4) monoclonal antibody specific for V. vulnificus in PBS. Add 50 µl to test wells. Control wells receive antibody with specificity other than V. vulnificus, tissue culture media, or PBS. Incubate at room temperature for 1 h. Wash plate 3 times with wash solution. To inquire about monoclonal antibody to V. vulnificus, contact E. L. Elliot.

Add conjugate. Dilute peroxidase-conjugated goat anti-mouse IgG with PBS. Add 50 µl to each well and incubate in dark at room temperature for 1 h. Wash 5 times.

Add substrate. Add 100 µl freshly prepared ABTS substrate solution to each well. Incubate about 10 min at room temperature, or until maximum color develops (usually less than 30 min). Compare negative controls to respective test wells for positive reactions. A well is usually considered positive if its optical density is 0.200 above that of negative control. An EIA plate reader is normally not required to differentiate reactions, but if used, read optical density at 410 nm.

3. V. vulnificus flagellar agglutination (27). Use latex beads coated with monoclonal antibodies (IgM or IgG isotype) to V. vulnificus H antigen (flagellar core protein) in flagellar agglutination test for presumptive identification of isolates from mCPC agar. The coagglutination reagent, prepared by Simonson and Siebeling (27), is available from E. L. Elliot.

   Inoculate alkaline peptone agar or other nonselective agar slants with suspect colonies and incubate 18-24 h at 30°C. Harvest growth by washing slant with Tris-EDTA-Triton X-100 (TET) buffer or formalinized PBS, pH 7.2-7.5. Hold cells in TET buffer at room temperature for 1 h to allow the Triton X-100 to remove sheath from flagellar cores. Formalinized PBS-suspended cells may be tested immediately.

   Use a micropipettor to place a 7-15 µl drop of suspended cells on glass slide and mix with equal volume drop of coagglutination reagent (antibody-coated on latex beads). Mix with wooden applicator stick and tilt slowly. Hold slide over indirect lighting and examine TET-suspended cells for agglutination in 10 s to 1 min. Formalinized PBS-suspended cells may require 2-3 min to agglutinate.

   Controls. For autoagglutination control, perform test with suspended cells and uncoated latex beads. For negative controls, mix coagglutination reagent with appropriate buffer and check for agglutination.

G. Gene probes

Gene probes (oligonucleotides) for V. cholerae enterotoxin (CTX A11), V. parahaemolyticus thermostable direct hemolysin (TDH-3), and V. vulnificus cytotoxin-hemolysin are available from Dr. Joseph Madden, FDA, 200 C St., SW, Washington, DC 20204, or from Fannie Harrell, HFR-MW460, MCI, MIN-DO, 240 Hennepin Ave., Minneapolis, MN 55401.

These probes are for genes associated with pathogenicity or species specificity. See Chapter 24 for gene probe methods.

H. Fatty acid analysis

Vibrio spp. may be identified by gas chromatographic analysis of cellular fatty acids. Warren Landry (FDA, Dallas District Office, FTS/255-5308 or 214-655-5308) has developed a computer library for identification of many bacterial species, including Vibrio spp., using the Hewlett-Packard Microbial Identification System. The equipment is not available in all FDA laboratories, but unusual Vibrio spp. isolates may be sent to the Dallas laboratory for study and confirmation.

Acknowledgments

This chapter is based on BAM, 6th ed., Chapter 12 (V. parahaemolyticus), by Robert M. Twedt and Gerard N. Stelma, Jr.; Chapter 13 (V. cholerae), by Joseph M. Madden, Barbara A. McCardell, and Brenda K. Boutin; and on methods for V. parahaemolyticus and V. vulnificus revised by Edna M. Sloan, Curtis J. Hagen, and Gayle A. Lancette.

References

1. Baross, J., and J. Liston. 1968. Isolation of Vibrio parahaemolyticus from the Northwest Pacific. Nature 217:1263-1264.

2. Baselski, V., R. Briggs, and C. Parker. 1977. Intestinal fluid accumulation induced by oral challenge with Vibrio cholerae or cholera toxin in infant mice. Infect. Immun. 15:704-712.

3. Baumann, P., and R.H.W. Schubert. 1984. Section 5. Facultatively anaerobic Gram-negative rods, Family II. Vibrionaceae, pp. 516-550. In: Bergey's Manual of Systematic Bacteriology, Vol. 1. J.G. Holt and N.R. Krieg (eds). Williams & Wilkins, Baltimore.

4. Brayton, P.R., R.B. Bode, R.R. Colwell, M.T. MacDonell, H.L. Hall, D.J. Grimes, P.A. West, and T.N. Bryant. 1986. Vibrio cincinnatiensis sp. nov., a new human pathogen. J. Clin. Microbiol. 23:104-108.

5. Davis, B.R., G.R. Fanning, J.M. Madden, A.G. Steigerwalt, H.B. Bradford, Jr., H.L. Smith, Jr., and D.J. Brenner. 1981. Characterization of biochemically atypical Vibrio cholerae strains and designation of a new pathogenic species, Vibrio mimicus. J. Clin. Microbiol. 14:631-639.

6. DePaola, A., C.A. Kaysner, and R.M. McPhearson. 1987. Elevated temperature method for recovery of Vibrio cholerae from oysters (Crassostrea gigas). Appl. Environ. Microbiol. 53:1181-1182.

7. Ewing, W.H., K.M. Tomfohrde, and P.J. Naudo. 1979. Isolation and identification of Vibrio cholerae and certain related vibrios: an outline of methods. Species 2:10-22.

8. Finkelstein, R.A., and S. Mukerjee. 1963. Hemagglutination: a rapid method for differentiating Vibrio cholerae and El Tor vibrios. Proc. Soc. Exp. Biol. Med. 112:355-359.

9. Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai, and T. Ueho. 1951. On the bacteriological examination of Shirasu food poisoning. J. Jpn. Assoc. Infect. Dis. 35:11-12.

10. Furniss, A.L., J.V. Lee, and T.J. Donovan. 1978. The vibrios. Public Health Service Monograph Series No. 11, Her Majesty's Stationery Office, London.

11. Grimes, D.J., J. Stemmler, H. Hada, E.B. May, D. Maneval, F.M. Hetrick, R.T. Jones, M. Stoskopf, and R. R. Colwell. 1984. Vibrio species associated with mortality of sharks held in captivity. Microbial Ecol. 10:271-282.

12. Han, G.K., and T.D. Khie. 1963. A new method for the differentiation of Vibrio comma and Vibrio El Tor. Am. J. Hyg. 77:184-186.

13. Hickman, F.S., J.J. Farmer, III, D.G. Hollis, G.R. Fanning, A.G. Steigerwalt, R.E. Weaver, and D.J. Brenner. 1982. Identification of Vibrio hollisae sp. nov. from patients with diarrhea. J. Clin. Microbiol. 15:395-401.

14. Honda, T., Y. Ni, and T. Miwatani. 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative Vibrio parahaemolyticus and related to the thermostable direct hemolysin. Infect. Immun. 56:961-965.

15. Iwanaga, M., and K. Yamamoto. 1985. New medium for the production of cholera toxin by Vibrio cholerae 01 biotype El Tor. J. Clin. Microbiol. 22:405-408.

16. Kaper, J., E.F. Remmers, and R.R. Colwell. 1980. A presumptive medium for identification of Vibrio parahaemolyticus. J. Food Prot. 43:956-958.

17. MacDonell, M.T., F.L. Singleton, and M.A. Hood. 1982. Diluent decomposition of use of API 20E in characterizing marine and estuarine bacteria. Appl. Environ. Microbiol. 44:423-427.

18. Madden, J.M., B.A. McCardell, and J.G. Morris, Jr. 1989. Vibrio cholerae, pp. 525-542. In: Foodborne Bacterial Pathogens, M.P. Doyle (ed). Marcel Dekker, New York.

19. Massad, G., and J.D. Oliver. 1987. New selective and differential medium for Vibrio cholerae and Vibrio vulnificus. Appl. Environ. Microbiol. 53:2262-2264.

20. Nishibuchi, M., S. Doke, S. Toizumi, T. Umeda, M. Yoh, and T. Miwatani. Isolation from a coastal fish of Vibrio hollisae capable of producing a hemolysin similar to the thermostable direct hemolysin of Vibrio parahaemolyticus. Appl. Environ. Microbiol. 54:2144-2146.

21. Oliver, J.D. 1989. Vibrio vulnificus, pp. 569-600. In: Foodborne Bacterial Pathogens. M.P. Doyle (ed). Marcel Dekker, New York.

22. Pavia, A.T., J.A. Bryan, K.L. Maher, T.R. Hester, Jr., and J.J. Farmer, III. 1989. Vibrio carchariae infection after a shark bite. Ann. Intern. Med. 111:85-86.

23. Sakazaki, R. 1979. Vibrio infections, pp. 173-209. In: Foodborne Infections and Intoxications, 2nd ed. H. Riemann and R. Bryan (eds). Academic Press, New York.

24. Sakazaki, R., and T. Shimada. 1986. Vibrio species as causative agents of foodborne infections, pp. 123-151. In: Developments in Food Microbiology--2. R.K. Robinson (ed). Elsevier Applied Science, New York.

25. Shantera, W.X., J.M. Johnston, B.R. Davis, and P.A. Blake. 1983. Disease from infection with Vibrio mimicus, a newly recognized Vibrio species. Ann. Intern. Med. 99:169-171.

26. Shimada, T., R. Sakazaki, and M. Oue. 1987. A bioserogroup of marine vibrios possessing somatic antigen factors in common with Vibrio cholerae O1. J. Appl. Bacteriol. 62:452-456.

27. Simonson, J.G., and R.J. Siebeling. 1986. Rapid serological identification of Vibrio vulnificus by anti-H coagglutination. Appl. Environ. Microbiol. 52:1299-1304.

28. Simpson, L.M., V.K. White, S.F. Zane, and J.D. Oliver. 1987. Correlation between virulence and colony morphology in Vibrio vulnificus. Infect. Immun. 55:269-272.

29. Smith, Jr., H.L., and K. Goodner. 1958. Detection of bacterial gelatinases by gelatin-agar plate methods. J. Bacteriol. 76:662-665.

30. Tamplin, M.L., A.L. Martin, A.D. Ruple, D.W. Cook, and C.W. Kaspar. 1991. Enzyme immunoassay for identification of Vibrio vulnificus in seawater, sediment, and oysters. Appl. Environ. Microbiol. 57:1235-1240.

31. Twedt, R.M. 1989. Vibrio parahaemolyticus, pp. 543-568. In: Foodborne Bacterial Pathogens. M.P. Doyle (ed). Marcel Dekker, New York.

32. Twedt, R.M., J.T. Peeler, and P.L. Spaulding. 1980. Effective ileal loop dose of Kanagawa-positive Vibrio parahaemolyticus. Appl. Environ. Microbiol. 40:1012-1016.

33. West, P.A., and R.R. Colwell. 1984. Identification and classification of Vibrionaceae--an overview, pp. 285-363. In: Vibrios in the Environment. R.R. Colwell (ed). John Wiley & Sons, New York.

34. West, P.W., P.R. Brayton, T.N. Bryant, and R.R. Colwell. 1986. Numerical taxonomy of vibrios isolated from aquatic environment. Int. J. Syst. Bacteriol. 36:531-543.

35. Yamamoto, K., Y. Takeda, T. Miwatani, and J.P. Craig. 1983. Evidence that a non-O1 Vibrio cholerae produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect. Immun. 41:896-901.