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Chapter 9: *Vibrio*

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Revision History

- Chapter 9 substantially rewritten and revised May 2004

Introduction

Members of the genus *Vibrio* are defined as Gram-negative, asporogenous rods that are straight or have a single, rigid curve. They are motile; most have a single polar flagellum, when grown in liquid medium. Most produce oxidase and catalase, and ferment glucose without producing gas (7). Three species, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, are well-documented human pathogens (54,78,79, 90,101). *V. mimicus* (24,103,111), is a recognized pathogen (103) with similar characteristics to *V. cholerae,* except an ability to ferment sucrose. Other species within the genus, such as *V. alginolyticus* (51), *V. fluvialis* (71), *V. furnissii* (15), *V. metschnikovii* (39,70), and *V. hollisae* (40) are occasional human pathogens (1,39, 96). *Vibrio* species account for a significant proportion of human infections from the consumption of raw or undercooked shellfish (96). A Florida study of illnesses from raw shellfish consumption reported the following species in descending order of frequency; *V. parahaemolyticus*, non-O1/O139 *V. cholerae, V. vulnificus, V. hollisae, V. fluvialis,* O1 *V. cholerae* (64,72).

A number of substantial changes have been made in this version of the *Vibrio* chapter including greater emphasis on molecular methods such as DNA colony hybridization and PCR for identification and characterization of pathogenic *Vibrio* spp. With the addition of these options, less emphasis has been placed on some of the older methods and in some case some sections requiring dangerous or difficult to obtain reagents (i.e. O/129 reagent) have been eliminated but may be mentioned in the text or tables. There have been considerable advances in molecular detection techniques such as real time PCR and as these methods are validated, they will be incorporated into the web version of this chapter.

V. cholerae

V. cholerae (6), the type species of the genus *Vibrio*, is the causative agent of cholera outbreaks and epidemics (34,54,126). Various biochemical properties and antigenic types characterize it. It can be differentiated from other *Vibrio* species, except *V. mimicus*, because its obligate requirement for sodium ion (Na^+) (6) can be satisfied by the trace amounts present in most media constituents. Cholera enterotoxin (CT) is the primary virulence factor of the disease cholera. A genetic pathogenicity island designated VPI (vibrio pathogenicity island), which contains most genes necessary to cause cholera was demonstrated to regulate the CT gene (55). Most *V. cholerae* strains recovered from epidemic cholera cases contain a common somatic antigen and include serogroup O1 (54). Over 150 known somatic antigenic types have

been identified. Strains that are agglutinable in Inaba or Ogawa serotypes of O1 antiserum are well-documented human pathogens. Until recently, only the O1 serogroup was associated with cholera epidemics. However, in 1993, a large outbreak of cholera occurred in India/Bangladesh from a new, until then unknown serogroup, O139 (3). Numerous cases were recorded in which patients had the typical symptoms of classical cholera, *cholera gravis,* previously only seen with the O1 serogroup. Except for the O antigen and the presence of a polysaccharide capsule, this serogroup is nearly identical to the seventh pandemic strain of *V. cholerae* (10). The O139 strain has become endemic in the Bengal region and is the cause of what may be known as the Eighth Cholera Pandemic (34,117).

V. cholerae strains that are identical to, or closely resemble, clinical strains in biochemical characteristics, but fail to agglutinate in either anti-O1 or -O139 sera are now referred to as *V. cholerae* non-O1/O139 (34,53,54). These serologically diverse strains are abundant in estuarine environments. Evidence indicates that non-O1/O139 strains are sporadically involved in cholera-like diarrheal disease (22,73,83,96,105), but rarely in outbreaks. Indeed, the permeability factor produced by a non-O1/O139 strain during an investigation of a cholera outbreak was found to be biologically and immunologically indistinguishable from CT. Some non-O1/O139 strains also are invasive, produce a heat stable toxin, and have caused septic infections in individuals with pre-disposing medical conditions (83,85,93,99). Most strains do not produce CT, the key difference between these and epidemic *V. cholerae* O1/O139.

V. mimicus

V. mimicus (24,102) has been associated with diarrhea following consumption of raw or undercooked seafood (96). Isolated from samples during a search for *V. cholerae, V. mimicus* can be differentiated from that closely related pathogen by sucrose nonfermentation. The organism will appear as green colonies on thiosulfate citrate bile salts sucrose (TCBS) agar and will grow in most common media without added NaCl. Virulence is poorly characterized, but some strains have been found to possess the cholera toxin gene (111), produce demonstrable CT in a tissue culture assay, and the *ctx* gene can be detected by PCR amplification.

V. parahaemolyticus

V. parahaemolyticus (36,81), the leading cause of bacterial diarrhea associated with seafood consumption in Florida (64) and probably the US and occasionally causes septicemia (96). It is a halophilic estuarine organism found in coastal waters of virtually all temperate regions (27,52,101). In temperate regions, a seasonal occurrence in shellfish and in human infections has been reported, the majority in the warmer months of the year. In subtropical regions such as Florida, illness can occur year round. All strains share a common H antigen, but, to date, 12 O (symatic) types and over 70 K (capsular) antigens have been described, though many other strains are untypable (81,101). Most clinical isolates of *V. parahaemolyticus* are differentiable from environmental strains by their ability to produce a thermostable direct hemolysin (TDH), termed the Kanagawa phenomenon (82,120). The *tdh* gene has been cloned and sequenced (86,87). DNA probes now are available to test for this virulence marker in *V. parahaemolyticus* isolates (42,77,87). A thermostable related hemolysin (TRH), which shares 60% homology with TDH, has also been associated with strains causing gastroenteritis (45,46). Presently, there is no in vitro test to detect TRH production. Many clinical strains of *V.*

parahaemolyticus, produce both TDH and TRH (8,106). Taniguchi *et al*. (123) described a thermolabile hemolysin, TLH, found in all *V. parahaemolyticus* strains, but not in other species. PCR procedures and gene probes have been developed to detect the *tlh*, *tdh* and *trh* genes in *V. parahaemolyticus* (8,37,49,76,77).

V. vulnificus

V. vulnificus (33), the leading cause of death in the US related to seafood consumption and nearly always associated with raw Gulf Coast oysters (90,104), resembles *V. parahaemolyticus* on TCBS agar, but can be differentiated by several biochemical reactions, including β-galactosidase activity (31). Epidemiological and clinical investigations have shown that *V. vulnificus* causes septicemia and death following ingestion of seafood or after wound infections originating from the marine environment (43,118,129). Recent gene probe assays (29,134), PCR procedures (41), fatty acid profiles (68) and enzyme immunoassays (31,122) have been developed to detect and identify this pathogen.

Other Species

The following species have also been isolated from human stools and/or from patients with gastroenteritis, with the consumption of shellfish as the predominant source of infection (96). *V. metschnikovii* differs from all other *Vibrio* species in lacking cytochrome oxidase (7). Some strains (biotype II) of *V. fluvialis* sp. nov. (now designated *V. furnissii*) produce gas during D-glucose fermentation (15). *V. hollisae* is a halophilic species that grows poorly, if at all, on TCBS agar which exhibits a delayed motility pattern (>48 hr) uncharacteristic of the other vibrios (7). A variant of the tdh gene virulence marker of pathogenic *V. parahaemolyticus* strains was detected in some *V. hollisae* strains (40).

Differentiation of Species

Table 1 presents the differential characteristics of the species most often associated with human illness related to seafood consumption. Tables can also be found in several publications, including Baumann and Schubert (7), Elliot *et al.* (31), McLaughlin (78) and West *et al.* (131).

Table 1. Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood*

	V. alginolyticus	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschnikovi i*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*	*A. hydrophilia* **	*P. shigelloides* **
TCBS agar	Y	Y	Y	Y	NG	Y	G	G	G	Y	G
mCPC agar	NG	P	NG	NG	NG	NG	NG	NG	Y	NG	NG
CC agar	NG	P	NG	NG	NG	NG	NG	NG	Y	NG	NG
AGS	KA	Ka	KK	KK	Ka	KK	KA	KA	KA	KK	nd
Oxidase	+	+	+	+	+	–	+	+	+	+	+
Arginine dihydrolase	–	–	+	+	–	+	–	–	–	+	+
Ornithine decarboxylase	+	+	–	–	–	–	+	+	+	–	+
Lysine decarboxylase	+	+	–	–	–	+	+	+	+	V	+
Growth in (w/v): 0% NaCl	–	+	–	–	–	–	+	–	–	+	+
Growth in (w/v): 3% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth in (w/v): 6% NaCl	+	–	+	+	+	+	–	+	+	+	–
Growth in (w/v): 8% NaCl	+	–	V	+	–	V	-	+	-	-	-
Growth in (w/v): 10% NaCl	+	–	–	–	–	–	–	–	–	–	–
Growth at 42°C	+	+	V	–	nd	V	+	+	+	V	+
Acid from: Sucrose	+	+	+	+	–	+	–	–	–	V	–
Acid from: D-Cellobiose	–	–	+	–	–	–	–	V	+	+	–
Acid from: Lactose	–	–	–	–	–	–	–	–	+	V	–
Acid from: Arabinose	–	–	+	+	+	–	–	+	–	V	–
Acid from: D-Mannose	+	+	+	+	+	+	+	+	+	V	–
Acid from: D-Mannitol	+	+	+	+	–	+	+	+	V	+	–
Acid from: ONPG	–	+	+	+	–	+	+	–	+	+	–
Acid from: Voges-Proskauer	+	V	–	–	–	+	–	–	–	+	–
Sensitivity to: 10 µg O/129	R	S	R	R	nd	S	S	R	S	R	S
Sensitivity to: 150 µg O/129	S	S	S	S	nd	S	S	S	S	R	S
Sensitivity to: Gelatinase	+	+	+	+	–	+	+	+	+	+	–
Sensitivity to: Urease	–	–	–	–	–	–	–	V	–	–	–

* Adapted from Elliot *et al.* (31)
** *Aeromonas hydrophila, Plesiomonas shigelloides*
Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin; AGS, arginine-glucose slant;
Y = yellow NG = no or poor growth S = susceptible nd = not done
G = green V = variable among strains R = resistant P = purple, V = variable
KK = Slant alkaline / Butt alkaline KA = Slant alkaline /Butt acidic, Ka = Slant alkaline/ Butt slightly acidic

Distribution and Sources of Contamination

V. cholerae

V. cholerae O1 is excreted in great numbers in the feces of cholera patients and convalescents (34,54). The disease is transmitted primarily by the fecal-oral route, indirectly through contaminated water supplies (30,78,80,116,126,130). Direct person-to-person spread is not common. Food supplies may be contaminated by the use of human feces as fertilizer or by freshening vegetables for market with contaminated water (30,57,58,80,94). Cholera outbreaks in several countries and the US are thought to have resulted from the consumption of raw, undercooked, contaminated, or recontaminated seafood. Toxigenic *V. cholerae* O1 is rarely isolated from US environments and foods and no isolations of serogroup O139 have been reported in this country. In contrast, non-O1/O139 strains are commonly isolated from estuarine water and shellfish (5,126). Evidence suggests that *V. cholerae* O1 is a component of the autochthonous flora of brackish water, estuaries, and salt marshes of coastal areas of the temperate zone, posing an ongoing hazard to public health (11,126). Various O1 strains have become endemic in many regions in the world, including Australia and the Gulf Coast region of the US (19,127).

V. parahaemolyticus

This organism is frequently isolated from coastal waters and seafood in temperate zones throughout the world. It is the most frequent cause of foodborne disease in Japan (89), where many residents eat raw fish. A number of common-source gastroenteritis outbreaks attributed to *V. parahaemolyticus* have occurred in the US (57), associated with oyster consumption (88,96). Some foods implicated in the US are crab, shrimp, and lobster, which unlike fish in Japan, typically were cooked before eating. Mishandling practices, such as improper refrigeration, insufficient cooking, cross-contamination, or recontamination are suspected in these outbreaks. Recently, consumption of raw oysters was associated with large outbreaks of *V. parahaemolyticus* gastroenteritis on the West Coast in 1997 (17), and in Texas and New York in 1998 (18). Clinical strains from the West Coast were urease positive and possessed both *tdh* and *trh* genes. The Texas and New York outbreaks were caused by a urease-negative O3:K6 serotype, possessing only *tdh*. This strain appears to have become pandemic and is the most prevalent strain in Asia (10,23,74,135).

V. vulnificus

The invasive species, *V. vulnificus*, the causative agent of septicemic shock (63,90,118), is a common organism in coastal waters of some areas of the US and other countries (60,90,122,124). It is reported to cause 20 to 40 U.S. cases each year of primary septicemia with a 50% mortality rate among individuals with liver disease and elevated serum iron levels (104). A review of cases has determined an association between septicemia and consumption of raw oysters, nearly all from Gulf Coast waters. This species has also been responsible for

wound infections in individuals who are associated with marine environments (90). This halophilic species will grow on or in many laboratory formulations of media that contain NaCl; a 0.5% minimum concentration is recommended. Although virulence is associated with a capsule, no reliable marker has been identified; most tests cannot distinguish clinical from environmental strains (79,108,132).

Other halophilic vibrios

Like *V. parahaemolyticus, V. cholerae*, and *V. vulnificus*; *V. alginolyticus, V. fluvialis, V. furnissii, V. metschnikovii*, and *V. hollisae* are recovered from brackish coastal waters, sediment, and sea life taken from the temperate estuarine environment (7). These species are normal components of that environment, appear on a seasonal basis, and have been associated with human illness (12,96).

General Considerations

Storage of Sample

The sample should be cooled immediately after collection (about 7° C to 10° C) , then analyzed as soon as possible. Direct contact with ice should be avoided to maximize survival and recovery of vibrios. Vibrios can be injured by rapid cooling, but grow rapidly in seafood at ambient temperatures (20,21). Despite the recognized fragility of the vibrios to extremes of heat and cold, their survival is enhanced under mild refrigeration (13,14,16,38,50,95). When frozen storage of the sample is required, a temperature of -80°C is recommended, if feasible (14).

Shellfish samples should be handled according to recommended procedures described by the American Public Health Association (4). Ten-to-twelve animals are pooled, aseptically shucked to a sterile blender jar, and blended at high speed for 90 sec. This composite is used to prepare dilutions using a NaCl-containing solution, such as PBS.

To facilitate the storage and further analysis of numerous isolates from a sample, the following procedure is recommended. This method allows for the gene probe analysis of many isolates obtained from a sample, in contrast to the minimal number that can be feasibly handled using traditional biochemical tests. A sterile 96-well microtiter plate is filled with 100 µl/well of APW. Numerous colonies of presumptive vibrios are picked from a selective agar plate using a sterile toothpick or wood transfer stick to individual wells. The inoculation pattern is recorded and the plate is incubated 3-5h or overnight at 35 ±2°C. A 48-prong replicator is used to replicate/transfer isolates in the wells to an agar plate for gene probe analysis. After replication, 100 µl TSB-1% NaCl-24% glycerol (TSG) is aseptically dispensed to each well. The plate is wrapped in a double layer of foil or plastic and placed in an ultra-low freezer, -72° to -80°C, for storage of cultures. When needed, the plate is partially thawed and the cultures from the well(s) transferred, or replicated to a new microtiter plate or tubed medium. Purity of the culture can be determined by streaking to an agar medium such as T_1N_3.

Genetic Based Techniques

These newer technologies have the advantage of more rapid detection and identification and are included for those laboratories with the proper equipment. PCR-based identification offers a one-day analysis (5,8,9,35,41,66,67,107,109,119,125), while gene probe procedures, including those presented in this chapter, are one-to-two day analyses (29,37,42,61,69,76,77,87,97,100,133,134,136,137). The traditional qualitative procedure and the most probable number (MPN) technique require four-to-seven days to complete (31). Alkaline phosphatase (AP)-labeled probes to identify the presence of *V. parahaemolyticus* and strains harboring the *tdh* gene, and detecting *V. vulnificus* in a sample are available commercially. One lot of commercially AP-labeled probe is enough to process approximately 200 filters. Inexpensive paper filters (Whatman 541) can be used for colony lifts.

Digoxigenin (dig)-labeled amplicon probes (97) are also presented for the three species of concern. Advantages of the dig-labeled probe procedure are: (a) can be prepared in-house; (b) inexpensive to prepare; (c) more reporter groups per probe molecule; (d) twice the number of copies of the probe prepared as the reverse compliment is also labeled, (e) probe solution can be used several times, (f) the hybridization and wash temperature is the same for all dig-probes, (g) the nylon membrane can be stripped of a probe and hybridized with an additional probe(s), and (h) using a nylon membrane allows for the transfer between agar surfaces, i.e., from a non-selective agar for resuscitating cells prior to moving to a selective and differential agar. Hybridization times are greater than with AP-labeled probes and nylon membranes are more expensive than paper filters

Recommended Controls

More than one plating medium should be used for vibrios because strains may vary in their growth characteristics. T_1N_3 agar works well for all vibrios relevant to human health. Positive and negative control strains should be used for all phenotypic and genotypic assays to ensure appropriate interpretation of the reactions.

Media, Reagents, Supplies and Equipment

A. **Media and Reagents**

1. Alkaline peptone water (APW) (M10)
2. AKI medium (M7)
3. Arginine glucose slants (AGS) (M16)
4. Blood agar (5% sheep red blood cells) (M20)
5. Casamino acids yeast extract (CAYE) broth (M34)
6. modified Cellobiose polymyxin colistin (mCPC) agar (M98)
7. Cellobiose colistin (CC) agar (M189)

8. Motility test medium-1% NaCl (M103)
9. Oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl in dH_2O) (R54)
10. Peptone-Tween-salt diluent (PTS) (90)
11. Phosphate buffered saline (PBS) (R59)
12. Polymyxin B disks, 50 U (Difco or equivalent) (R64)
13. Saline soln - 0.85% in dH_2O (R63)
14. 2% NaCl soln (R71)
15. Sodium desoxycholate - 0.5% in sterile dH_2O (R91)
16. Thiosulfate citrate bile salts sucrose (TCBS) agar [M147]
17. T_1N_1 and T_1N_3 agars (1% tryptone and either 1% or 3% NaCl) (M163)
18. T_1N_0, T_1N_3, T_1N_6, T_1N_8, T_1N_{10} broths (M161)
19. Tryptic soy agar-magnesium sulfate- 3% NaCl (TSAMS) (32) Trypticase (or tryptic) soy broth (TSB) , agar (TSA)(M152) (with added NaCl, 2%)
20. TSB-1% NaCl-24% glycerol
21. Urea broth (M171) (or Christensen's urea agar (M40) with added NaCl (2%) (R71)
22. *V. cholerae* polyvalent O1 and O139 antiserum
23. VET-RPLA TD920A enterotoxin detection kit (Oxoid, Inc.)
24. *Vibrio parahaemolyticus* sucrose agar (VPSA) (M191)
25. *Vibrio vulnificus* agar (VVA) (M190)
26. API 20E diagnostic strips and reagents (BioMerieux)

B. **Probe Reagents, Equipment and Materials Required**

1. Shaking water bath(s) capable of up to 65°C. (temps needed, 42, 54, 55 and 65°C)
2. Shaker platform at room temperature
3. Microwave
4. Long wave UV light box or UV Crosslinker (254 nm wavelength)
5. Heat tolerant bags (and sealer) or plastic tubs with lids (300-500 ml capacity)

6. 96 well microtiter plates with lids
7. 8 or 12 channel micro-pipetter
8. 48 prong replicator
9. Whatman 541 filters, 85 mm (special order for this dia, 1541-085 from Whatman)
10. Whatman #3 or equivalent absorbent filter or pad
11. Nylon membranes (MagnaGraph Transfer membrane)(positive charge), 82 mm (Osmonics, Inc, Westboro, MA, gridded-NJOHG08250, plain-NJOHY08250)
12. Fiberglass mesh screens, household window screen available at hardware stores (59)
13. Sterile hockey sticks
14. Sterile toothpicks or wood applicator sticks
15. Glass petri dishes, 100 mm
16. Lysis soln (0.5 M NaOH, 1.5 M NaCl) (Maas I) - (R94) add to reagents list
17. Neutralizing soln (1.0 M Tris-HCl, pH 7.0 in 2.0 M NaCl) for nylon membranes (Maas II) (R95)
18. 2M ammonium acetate buffer (for AP-labeled probe and 541 filters) - (R1) modify reagent 1 to include this.
19. 1× SSC, 5× SSC, 20× SSC (standard saline citrate) - (R77) edit to add 1,5,20
20. 1× SSC - 1% SDS (sodium dodecyl sulfate) and 3× SSC- 1% SDS - (R93) add new reagent
21. 10% Sarkosyl soln (N-lauroyl-sarcosine,sodium salt) - (R96) add new reagent
22. 10% SDS soln (sodium dodecyl sulfate) - (R92) add new
23. 1M Tris, pH 7.5 (Trizma base; Sigma Cat. No. T1503)
24. 1M Tris, pH 9.5 (Trizma base; Sigma Cat. No. T1503)
25. 3M NaCl
26. 1M $MgCl_2$
27. Proteinase K stock solution (20 mg/ml)
28. Hybridization solution (BSA, SDS, PVP in 5× SSC) (for AP-labeled probe)

29. NBT/BCIP color reagent [nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate], toluidine salt, Roche Diagnostics Cat. No. 1697471 (for colorimetric detection)

30. Dig buffers 1, 2, 3 and 4 (97)

31. 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

32. Blocking reagent (Roche Diagnostics Cat. No.1096176)

33. Wash soln A and B (97)

34. Anti-Dig AP [Anti-digoxigenin alkaline phosphatase, Fab fragments] (Roche Diagnotics Cat. No. 1093274)

35. dig-11-dUTP (Roche Diagnostics Cat. No. 1093088)

36. CSPD Roche Diagnostics Cat. No. 1755633 (for chemiluminescent detection)

Precautions

A good selective enrichment broth has not been developed for *V. cholerae*. However, due to its rapid generation time, short incubation periods are effective for isolation. APW provides suitable enrichment for incubation periods of 6 to 8 h, but other competing microflora may overgrow *V. cholerae* during longer enrichment periods for certain types of samples. Overnight periods (16 to 18 h), although not desirable, have been used to facilitate sample analysis during work hours. If the product was subjected to a processing step, i.e. heating, freezing, drying, or low densities are expected, incubation overnight is recommended to thoroughly resuscitate injured cells. The incubation of raw oyster samples in APW at 42°C for 6 to 8 h has proven effective for isolation of *V. cholerae* and is recommended (26). However, DePaola and Hwang (28) found that enrichment incubation for 18 to 21 h, instead of 6 to 8 h, gave a higher recovery of O1 *V. cholerae* when low inocula were used. Because of these considerations, it is recommended to streak APW enrichments both after 6 to8 h and after overnight incubation. It is also recommended for raw oysters to use a 1:100 ratio of oyster to APW (28).

Procedures

V. cholerae

A. Enrichment and plating

1. Weigh 25 g of sample into a tared jar (capacity approximately 500 ml). Products such as seafood or vegetables may be blended or cut into small pieces with sterile scissors.

2. Add 225 ml APW to jar. Thoroughly mix the sample or blend 2 min at high speed.

3. Incubate APW at 35 ±2°C for 6 to 8 h. Re-incubate the jar overnight if the sample had been processed in some way. For analysis of raw oysters, include a second tared flask with 25 g of product plus 2475 ml APW. This flask should be incubated 18 to 21 h at 42 ±0.2 °C in a water bath (28,31). An enumeration technique by most probable number (MPN) may also be performed if desired.

4. Prepare dried plates of TCBS agar. Modified-CPC or CC agars may also be included.

5. Transfer a 3-mm loopful from the surface pellicle of APW culture to the surface of a dried TCBS plate (and mCPC or CC), and streak in a manner that will yield isolated colonies.

6. Incubate TCBS overnight (18 to 24 h) at 35°±2°C. Incubate mCPC and CC overnight at 39-40°C; if a 39-40°C incubator is not available then 35-37°C is usually adequate as selectivity is determined primarily by antibiotics in formulation than by high temperature.

7. Typical colonies of *V. cholerae* on TCBS agar are large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries.

8. Typical colonies of *V. cholerae* on mCPC or CC agar are small, smooth, opaque, and green to purple in color, with a purple background on extended incubation.

9. For biochemical identification, colonies from crowded plates must be streaked to a non-selective agar (T_1N_1, T_1N_3, or TSA-2% NaCl agar) for purity. Incubate overnight at 35 ±2° C and proceed with identification using a single isolated colony.

10. Subculture three or more typical colonies from each plating medium to T_1N_1 agar slants or motility test medium stabs. Incubate slants or stabs overnight at 35° ±2°C.

B. Screening and Confirmation

1. Arginine glucose slant (AGS). Inoculate each suspect T_1N_1 culture to AGS by streaking the slant and stabbing the butt. Incubate AGS with loose cap overnight at 35° ±2°C. *V. cholerae* and *V. mimicus* cultures will have an alkaline (purple) slant and an acid (yellow) butt, as arginine is not hydrolyzed. No gas or H_2S is produced.

2. Salt tolerance. From T_1N_1 culture, lightly inoculate one tube each of $T_1N_0$and T_1N_3 broths. Incubate tubes overnight at 35° ±2°C. *V. cholerae* and *V. mimicus* cultures will grow without NaCl.

3. String test. The string test (110) is a useful presumptive test for suspected *V. cholerae* as all strains are positive. Emulsify a large colony from a T_1N_1 agar culture in a small drop of 0.5% sodium desoxycholate in sterile dH_2O. Within 60 sec the cells lyse (loss of turbidity) and DNA strings when a loopful is lifted (up to 2 to 3 cm) from the slide.

4. Oxidase reaction. Transfer the overnight T_1N_1 growth using a platinum wire (nichrome wire should not be used) or wood applicator stick to a filter paper saturated with oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl). A dark purple color developing within 10 sec indicates a positive test growth. Alternatively, add a drop of reagent to the growth on a T_1N_1 slant or agar plate. *V. cholerae* and *V. mimicus* are oxidase positive.

5. Serologic agglutination test. Serotyping of suspect *V. cholerae* cultures passing the string test using somatic or O antigens gives important epidemiological evidence. Two major serotypes of serogroup O1, Ogawa and Inaba, and serogroup O139 are recognized as human pathogens. The two serotypes of O1 are seen in both the classical *V. cholerae* and the El Tor biotypes. The O139 serogroup resembles only the El Tor biotype.

    a. For each culture, mark off three sections (with wax pencil) about 1 × 2 cm on the inside of a glass petri dish or on a 2 × 3-inch glass slide and add one drop of 0.85% saline solution to the lower part of each marked section. With a sterile transfer loop or needle, emulsify the T_1N_1 culture in the saline solution for one section, and repeat for the other section. Check for agglutination.

    b. Add a drop of polyvalent *V. cholerae* O1 antiserum to one section of emulsified culture and mix with a sterile loop or needle. Add a drop of anti-O139 to a separate section. (Third section)

    c. Tilt the mixture back and forth for one min and observe against a dark background. A positive reaction is indicated by a rapid, strong agglutination in a clear background.

    d. If positive, test separately with Ogawa and Inaba antisera. The Hikojima serotype reacts with both antisera.

    e. Antibodies to the Inaba and Ogawa, and group O1 antigen are commercially available (i.e. Columbia Diagnostics Inc., Springfield, VA). Similarly, O139 antiserum is commercially available.
    Results of non-agglutinable cultures should be reported as non O1/O139 *V. cholerae*.

C. Biochemical tests

Table 2 presents the minimal number of characters needed to identify *V. cholerae* strains. The ability of *V. cholerae* to grow in 1% tryptone without added NaCl differentiates it from other sucrose-positive vibrios. The API 20E diagnostic strip has been used successfully for identification and confirmation of isolates (92). The microtiter plate system for storage of suspect isolates can be used here.

D. Differentiation of El Tor and Classical biotypes.

Although the Classical biotype is rarely encountered, the following are optional tests to differentiate them from the El Tor biotype:

1. Beta-hemolysis. The most common means of differentiating the biotypes of O1 *V. cholerae*, and perhaps the easiest, is to determine β-hemolytic ability on sheep blood agar. El Tor strains are β-hemolytic, while classical strains do not produce a hemolysin. Inoculate a blood agar plate with test cultures by spotting to the surface and incubate 18-24 h at 35° ±2°C. Beta-hemolysin can be determined by a clear zone around the growth of the culture.

2. Polymyxin-B sensitivity. Streak a suspect culture to a dry T_1N_1 agar plate and place a 50 unit disc of polymyxin-B on the surface. Invert the plate, incubate overnight at 35° ±2°C, and record the result. Classical strains are sensitive (>12 mm zone); El Tor strains are resistant. If the suspect culture grows on mCPC agar, which contains polymyxin B, it is considered to be of the El Tor biotype.

E. Determination of enterotoxigenicity

Most strains of *V. cholerae* isolated from foods or the environment do not produce cholera toxin, (CT) and are not considered virulent. Isolates identified as *V. cholerae* or *V. mimicus* should be tested for the production of CT or the *ctx* gene (111).

1. Y-1 mouse adrenal cell assay (98). CT has been shown to stimulate the enzyme adenylate cyclase with the production of cyclic adenosine monophosphate that ultimately influences several cellular processes. In the Y-1 cell assay, CT promotes the conversion of elongated fibroblast-like cells into round refractile cells.

   The maintenance and passage of cell cultures, preparation of microtiter assay plates and conduct and interpretation of assay are carried out as in Chapter 4- *Escherichia coli* of this manual.

   a. Inoculate test cultures from T_1N_1 slants to tubes of CAYE broth and incubate overnight at 30° ±2°C.

   b. Inoculate a 10 ml portion of CAYE broth in a 50 ml Erlenmeyer flask from each stationary culture; incubate for 18 hr with shaking. Centrifuge each test culture; filter the supernatant through a 0.22 µm filter. Refrigerated filtrates may be stored for up to 1 week.

   c. Add aliquots of 25 µl from each filtrate, both unheated and heated to 80°C for 30 min, to wells of the microtiter assay plate. In addition to filtrates from known toxigenic and nontoxigenic cultures, add 0.025 ml aliquots from preparations containing 1.0 and 0.1 ng CT/ml. Suppression of cell rounding by treatment of test filtrates with anti-CT serum is an advisable control for nonspecific reactions.

2. Immunoassay for CT. A commercially available immunoassay has been developed to detect the presence of CT in cultural filtrates of *V. cholerae* and *V. mimicus* (VET-RPLA, Oxoid, Inc., Ogdensburg, NY).

   a. Inoculate test cultures into AKI medium and incubate at 35 ±2°C 18 h with shaking at 100 rpm. Centrifuge 5 to 7 ml of culture at 8,000 × g for 10 min. Filter sterilize the supernatant through a 0.2 µm filter or used as is.

Test the supernatant or filtrate following the manufacturer's protocol using conical 96 well microtiter plates. Incubate the plate overnight, undisturbed at room temperature.

3. Other toxins

   The significance of other toxins in human pathogenicity is poorly understood and these assays are not recommended for routine analysis. Madden *et al.* (73) also demonstrated clinical isolates that were pathogenic for infant rabbits. A heat-labile cytolysin produced by *V. cholerae* non-O1/O139 was found by McCardell *et al*. (75) to be cytotoxic to Y-1 mouse adrenal and Chinese hamster ovary cells, to be rapidly fatal upon intravenous injection into adult mice, and to cause fluid accumulation in rabbit ileal loops (112). Cultures may be tested for heat stable enterotoxin (ST) (75,121) or cytotoxin (83,100) if desired.

F. Genotypic detection of the cholera toxin gene by polymerase chain reaction (67)

The CT gene may be present in strains of *V. cholerae* and *V. mimicus*, but not expressed under experimental conditions. Thus a genotypic assay such as PCR amplification of the *ctx* gene is recommended. This procedure offers a more rapid result and is less complicated than phenotypic assays.

1. Cholera toxin PCR primers, 10 pmol/µl stock solutions.

   a. Forward 5'-tga aat aaa gca gtc agg tg-3'

   b. Reverse 5'-ggt att ctg cac aca aat cag-3'

   The PCR product is a 777 bp fragment.

2. APW enrichment. From sample preparation above of the 6-21 h incubation, prepare a crude lysate for PCR by boiling 1 ml of APW enrichment mixture in a 1.5 ml microcentrifuge tube for 10 min. Lysate can be used immediately for PCR or stored at -20°C until use. For suspect *V. cholerae* and *V. mimicus* isolates and control cultures, inoculate 1 ml vol of APW, incubate 18 h at 35 ° ±2°C and proceed with boiling step.

3. To minimize cross-contamination of PCR reagents, it is recommended that a PCR master mix be prepared and aliquots stored frozen (-20°C) until use. Master mixes contain all necessary reagents except *Taq* polymerase and the lysate (template) to be amplified. The final reaction contains: 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 200 µM each of dATP, dTTP, dCTP, dGTP; 2 to 5% (v/v) APW lysate (template); 0.5 µM of each primer and 2.5 U *Taq* polymerase per 100 µl reaction. Volumes of 25 to 100 µl may be used. Add *Taq* polymerase to the master mix and add template upon distribution to 0.6 ml microcentifuge tube reaction vessels. Some thermocyclers may require a mineral oil overlay (50-70 µl). The following thermocycler conditions should be used:

Thermocycler conditions	Time (min)	Temperature (°C)
Initial denaturation	3	94
Denaturation	1	94
Primer annealing	1	55
Primer extension	1	72
Final extend	3	72

No. cycles: No more than 35

4. Agarose gel analysis of PCR products. Mix 10 µl PCR product with 2 µl 6× loading gel and load sample wells of 1.5 to 1.8% agarose gel containing 1 µg/ml ethidium bromide submerged in 1× TBE. Use a constant voltage of 5 to 10 V/cm. Illuminate gel with a UV transluminator and visualize bands relative to molecular weight marker migration. The primers listed give a 777 bp fragment of *ctxAB*. Polaroid photographs can be taken of the gel for documentation. Positive and negative culture controls and reagent control should be included with each PCR run.

   Probes have been developed to also detect the presence of *ctxAB* (133) A dig-labeled probe can also be prepared of the PCR amplification product for detection of the *ctxAB* gene using colony hybridization. The preparation of the probe, the hybridization conditions for colony blots of suspect isolates and wash protocol follow those outlined in the technical literature of Roche Diagnostics, Indianapolis, IN(97).

5. Final report.

   The final report for *V. cholerae* should include biochemical and serological identification of the isolate and enterotoxicity results. The minimal number of characters to identify the species are presented in Table 2.

   Table 2. Minimal Number of Characters needed to Identify *V. cholerae* and *V. parahaemolyticus* Strains

	Positive Reaction	Percentage
Gram-negative, asporogenous rod	+	100
oxidase	+	100
String	±	100
L-lysine decarboxylase	+	100
L-arginine dihydrolase	-	0
L-ornithine decarboxylase	+	98.9
growth in 1% tryptone broth[a]	+	99.1[b]/0[c]

[a] No sodium chloride added.
[b] *V. cholerae* (and *V. mimicus*)
[c] *V. parahaemolyticus*
From Hugh and Sakazaki (48)

Other Vibrios

V. parahaemolyticus

Three analytical schemes for enumerating *V. parahaemolyticus* are presented. The first is the MPN procedure commonly used by many laboratories. In addition, this procedure is nearly identical for enumeration of *V. vulnificus*. The second is a membrane filtration procedure using hydrophobic grid membrane filter (HGMF) (32). The third is a direct plating method using DNA probes for identification of the total *V. parahaemolyticus* population (76) and pathogenic (TDH containing) strains (77). In addition, a TRH gene probe procedure and a PCR confirmation analysis (8) are also included.

A. Seafood samples: Enrichment, isolation, and enumeration.

1. Weigh 50 g of seafood sample into a blender. Obtain surface tissues, gills, and gut of fish. Shellfish samples include meat and liquor. Normally 12 animals are pooled, blended at high speed for 90 sec and 50 g of homogenate used for analysis. For crustaceans such as shrimp, use the entire animal if possible; if it is too large, select the central portion including gill and gut. Note: same for *V. vulnificus*

2. Add 450 ml PBS dilution water and blend for 1 min at 8,000 RPM. This constitutes the 1:10 dilution.

   Prepare 1:100. 1:1000, 1:10,000 dilutions or higher, if necessary, in PBS.

   a. For molluscan shellfish, pool 12 animals. Blend 90 sec with an equal vol of PBS (1:2 diln) (4). Prepare a 1:10 dilution by transferring 20.0g (weighing is recommended because air bubbles in the 1:2 dilution prevent accurate volumetric transfer) of the 1:2 to 80 ml of PBS. Additional 10-fold dilutions can be prepared volumetrically (i.e. 1ml of 1:10 to 9.0ml of PBS for a 1:100 dilution.

   b. For product that has been processed, i.e. heated, dried, frozen, inoculate 3 × 10 ml portions of the 1:10 dilution into 3 tubes containing 10 ml of 2× APW. This represents the 1 g portion. Similarly, inoculate 3 × 1 ml portions of the 1:10, 1 :100, 1: 1000, and 1 :10,000 dilutions into 10 ml of single-strength APW. If high numbers of *V. parahaemolyticus* are expected, the examination may start at the 1:10 dilution of product.

3. Incubate APW overnight at 35 ±2°C.

4. Streak a 3-mm loopful from the top 1 cm of APW tubes containing the three highest dilutions of sample showing growth onto TCBS (and mCPC or CC agars for *V. vulnificus* isolation)

5. Incubate TCBS plates at 35 ±2°C (and mCPC or CC plates preferably at 39-40 °C or 35-37°C if 39-40°C is not available) overnight.
   V. parahaemolyticus appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies

are, large, opaque, and yellow. Most strains of *V. parahaemolyticus* will not grow on mCPC or CC agar. If growth occurs, colonies will be green-purple in color due to lack of cellobiose fermentation.
Purify isolates as described previously and inoculate a microtiter plate for freezer storage.

B. Screening and Confirmation

1. Biochemical identification of isolates. Unless otherwise specified, all media in this section are prepared to contain 2% or 3% NaCl. The API 20E diagnostic strip can be alternatively used here (92). Prepare a cell suspension of the suspect cultures in 2% NaCl for the API 20E.

    a. Screen suspect cultures of *V. parahaemolyticus* (and *V. vulnificus*), using AGS, and T_1N_0 and T_1N_3 broths as described previously. Incubate tubes at 35 ±2°C for 18-24 h.

    b. Transfer two or more suspicious colonies from TCBS agar with a needle to arginine glucose slant (AGS). Streak the slant, stab the butt, and incubate with the cap loose overnight at 35 ±2°C. Both *V. parahaemolyticus* and *V. vulnificus* produce an alkaline (purple) slant and an acid (yellow) butt (arginine dihydrolase negative), but no gas or H_2S in AGS.

    c. For TSB and TSA slants (supplemented with 2% NaCl), inoculate both media and incubate overnight at 35 ±2°C. These cultures provide inocula for other tests as well as material for the Gram stain and for microscopic examination. Both *V. parahaemolyticus* and *V. vulnificus* are oxidase positive, Gram-negative, pleomorphic organisms exhibiting curved or straight rods with polar flagella.

    d. Inoculate a tube of motility test medium by stabbing the column of the medium to a depth of approximately 5 cm. Incubate overnight at 35 ±2°C. A circular outgrowth from the line of stab constitutes a positive test. *V. parahaemolyticus* and *V. vulnificus* are motile.

    e. *V. parahaemolyticus* and *V. vulnificus* will only grow in T_1N_3 but not in T_1N_0. Only the salt-requiring cultures need to be tested further.

    Only motile, Gram-negative rods that produce an acid butt and an alkaline slant on AGS, do not form H_2S or gas, and are salt-requiring require further examination.

    f. The identifying characteristics of *V. parahaemolyticus* and *V. vulnificus* are presented in Table 1

    Biochemically, *V. parahaemolyticus* and *V. vulnificus* are phenotypically similar, but can be differentiated by differences of the ONPG, salt-tolerance, cellobiose and lactose reactions (Table 1). By using selected biochemical traits, *V. parahaemolyticus* and *V. vulnificus* can be

distinguished from most interfering marine vibrios and other marine microorganisms.

All *V. parahaemolyticus* isolates should be tested for the presence of urease, by either using urea broth supplemented with 2% NaCl or on Christensen's urea agar supplemented with NaCl, 2% final conc or using the API 20E. Clinical strains from the US West Coast and from Asian countries have been predominantly urease positive. Urease production is correlated with the presence of the *tdh* and/or *trh* genes (2, 49, 62, 88, 91, 114, 115). The urease reaction is a valuable screening test for potentially pathogenic strains (62).

Inoculate urea broth-3%NaCl with a heavy inoculum of culture or spot the culture to surface of Christensen's-urea-NaCl agar plate or slant. Incubate 35 ±2°C 18-24 h.

Production of urease is determined by a pink (alkaline) color to the medium.

Negative cultures should be incubated an additional 24 h for the rare, slow urease producing strains.

When the colonies are finally identified biochemically as *V. parahaemolyticus* refer to the original positive dilutions in the enrichment broth and apply the 3-tube-MPN tables (Appendix 2) for final enumeration of the organism.

g. Alternatively, isolates can be identified as *V. parahaemolyticus* or *V. vulnificus* by DNA probe hybridization or PCR as described in the following sections.

2. Hydrophobic grid membrane filtration enumeration procedure (HGMF) (25, 32)

The apparatus, filters, and specific instructions may be obtained from QA Laboratories, San Diego, CA.

a. Prepare a 1:10 dilution of seafood sample with peptone tween-salt diluent (PTS), and blend 60 sec at high speed. Filter 1.0 ml or other volume of homogenate through HGMF using sterile diluent as a carrier. With forceps, aseptically transfer the HGMF from the filtration apparatus to the surface of a dry tryptic soy agar magnesium sulfate NaCl plate (TSAMS) M152. Incubate 4 h at 35 ±2°C. Ten-fold dilutions may be prepared if high levels of *V. parahaemolyticus* are expected.

b. With forceps, aseptically transfer the HGMF from the TSAMS to the surface of a dry *V. parahaemolyticus* sucrose agar (VPSA) plate M191. Invert plate and incubate at 42°C for 18 to 20 h.

c. On VPSA, *V. parahaemolyticus* colonies will be green to blue filling at least one-half of the grid square. This is a presumptive enumeration. At least five representative colonies must be identified. Other growth will

normally be yellow due to sucrose fermentation. Confirmed squares must be multiplied for total typical colonies and the MPN/g of seafood calculated. For example if 3 of 5 presumptive colonies are biochemically confirmed as *V. parahaemolyticus* , then the total number of presumptive colonies should be multiplied by 0.6 to estimate the *V. parahaemolyticus* density. *V. vulnificus* colonies will also be blue/green in color. DNA probes can differentiate the species (29,61,76,134).

3. Serologic typing (47,81)

V. parahaemolyticus possesses three antigenic components: H, O, and K. The H antigen is common to all strains of *V. parahaemolyticus* and is of little value in serotyping. The K, or capsular antigen, may be removed from the bacterial body by heating the isolate for 1 or 2 hr at 100°C. This process exposes the O, or somatic, antigen, which is thermostable. Since the K antigen masks the O antigen, it is necessary to remove the former by heating before performing the O agglutination tests.

There are 12 O group and over 70 known K antigens (47). Five of the K antigens have been found to occur with either of two O group antigens; therefore, there are 76 recognized serotypes (Table 3). Serologic tests by themselves are not used to identify *V. parahaemolyticus* because of cross-reactions with many other marine organisms. However, during investigations of foodborne outbreaks, serologic tests become a valuable epidemiologic tool.

Table 3. Antigenic scheme of *V. parahaemolyticus* (1986)[a]

O group	K type
1	1,25,26,32,38,41,56,58,64,69
2	3,28
3	4,5,6,7,27,30,31,33,37,43,45,48,54,57,58,59,65
4	4,8,9,10,11,12,13,34,42,49,53,55,63,67
5	5,15,17,30,47,60,61,68
6	6,18,46
7	7,19
8	8,20,21,22,39,70
9	9,23,44
10	l9,24,52,66,71
11	36,40,50,51,61
12	52
Total 12	65

[a] The antigenic scheme was first established by Sakazaki *et al*. (101) and later extended by the Commission of the Serotyping of *V. parahaemolyticus* (Japan); K antigens, 2,14,16,29,35, and 62 were excluded by the Commission (47). K types 4,5,6,7,8,9, and 19 occur with more than one O group.

V. parahaemolyticus diagnostic antiserum kits are produced commercially in Japan and available from Nichimen Co., 1185 Avenue of the Americas. 31st Floor, New York, NY 10036; (212) 719-1000 or Accurate Chemical and Science Corp, San Diego, CA 800-255-9378. Because the antiserum is expensive, it is not recommended for most laboratories. CDC has serotyping capability.

4. Determining pathogenicity

Kato *et al*. (56) showed that *V. parahaemolyticus* isolates from the stools of patients with enteric infections are hemolytic on a special high-salt human blood agar, whereas *V. parahaemolyticus* isolates from seafood and marine water usually are not. Wagatsuma (128) later modified this special agar to avoid confusion with the regular normal hemolytic activity of *V. parahaemolyticus* on conventional 5 % sheep blood agar. The special agar was named Wagatsuma agar and the special hemolytic response the Kanagawa phenomenon. Freshly drawn human, dog or sheep blood is used in preparation of the agar.

The correlation has been well established that *V. parahaemolyticus* strains that cause illness in humans are almost always Kanagawa-positive and isolates recovered from seafood are almost always Kanagawa-negative (81,82,101,102,106). In addition, extensive investigation in animal models suggests that the Kanagawa hemolysin is the primary virulence factor in *V. parahaemolyticus* (82,120). The Kanagawa test, or hybridization with the *tdh* gene probe provides reliable information on the presence of pathogenic strains isolated from foods. Due to the difficulty of obtaining fresh blood and the strong correlation between Kanagawa phenomenon and presence of the *tdh* gene, it is recommended to use DNA probe methods described in this chapter to determine potential virulence of *V. parahaemolyticus* isolates instead of the Kanagawa phenomenon.

a. Genotypic detection of hemolysin genes of *V. parahaemolyticus*.

Alkaline phosphatase- and digoxigenin-labeled DNA probes can be used for the identification of *V. parahaemolyticus* . A thermolabile hemolysin gene, *tlh*, has been found in all strains of *V. parahaemolyticus*, but not in other species (123) and DNA probes have been used for identification. Two DNA probe procedures that have been shown to be equivalent are presented. DNA probes have also been constructed to detect the thermostable direct hemolysin, *tdh* (87) and thermostable related hemolysin, *trh* (46), genes that are associated with pathogenic strains.

An alkaline phosphatase-labeled (AP) *tlh* probe (76) is commercially prepared for use with Whatman 541 colony lifts. The hybridization and detection procedure for the AP-*tlh* and AP-*tdh* probes (77) are presented below, using a hybridization and wash temperature of 54°C. Digoxigenin-labeled probes for *tlh* and *trh* were constructed of PCR amplification

products using the primer sets reported by Bej *et al*. (8). The *tdh* probe was constructed using a primer set based on the oligonucleotide probes of Nishibuchi *et al*. (87), using *tdh1* as the forward primer and the reverse compliment of *tdh4 (tdh4c)* as the reverse primer. The probes are labeled with digoxigenin during amplification according to the procedure described by Roche Diagnostics(97). These amplicons are of the following sizes; 450 bp *tlh*, 424 bp *tdh* and 500 bp *trh*.

1. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh* and AP-*tdh*) (76,77)

   Store probes in the refrigerator for one-to-two years; do not freeze.

   Probe sequences are:

Species specific thermolabile hemolysin (*tlh*)	AP-labeled 5'Xaa agc gga tta tgc aga agc act g 3' (where X is the AP-label)
Thermostable direct hemolysin (*tdh*), the Kanagawa hemolysin,	AP-labeled 5'Xgg ttc tat tcc aag taa aat gta ttt g 3'

   Probes can be purchased from DNA Technology ApS, Science Park Aarhus, Gustav Wledsd Vej 10, DK-8000, Aarthus C, Denmark. Phone 45 86 20 33 88, Fax 45 86 20 21 21, e-mail oligo@dnatech.aau.dk.

2. Sample preparation and dilutions are the same as with the MPN procedure. In addition, the preparation of sample and the hybridization conditions are the same for the simultaneous enumeration of *V. vulnificus*, except plating to VVA M190 and a 55°C hybridization temperature (29). Just before use, thoroughly dry T_1N_3 M161 (and VVA) agar plates inverted with lids cracked open for 1 h at 35°C. This is a non-selective agar.

   i. Pool 10-12 oyster meats and homogenize in equal part, by weight, with PBS for 90 sec at high speed (1:1 dilution) (4).

   ii. Weigh 0.20 g of this oyster:PBS (1:2) homogenate directly from blender (represents 0.1 g or -1 dilution of oyster tissue) onto T_1N_3 plate using balance with 0.01g sensitivity to tare plate. Pipet 100 µl of -1, -2 and -3 dilutions onto T_1N_3 plates with the dilutions. For shellfish harvested from December through March, plating -1 and -2 dilutions is adequate, while during May through October, summer months,-1, -2, and -3 dilutions are adequate for plating. The detection level is 10 CFU/g.

iii. For seafood other than oysters, the initial dilution of 1:10 should be used, because of the product debris from homogenizing. Inoculate the surface of T_1N_3 with 100 µl of this 1:10 dilution of sample. Thus the detection level will be 100 CFU/g.

iv. Use sterile hockey sticks to spread inoculum evenly onto T_1N_3 agar plates. Dry plates and uniform distribution of inoculum are essential for adequate colony isolation.

v. Incubate plates 18-24 h at 35 ±2° C. All plates should be used for colony lifts and hybridization unless there is confluent growth.

3. Filter preparation

i. Overlay T_1N_3 (and VVA) plates with labeled (sample number, dilution) #541 Whatman filters (85 mm) for 1 to 30 min. For plates to be used for *tdh* detection, mark filter and plate for alignment and subsequent colony recovery. Store plate in refrigerator. Transfer filters with colony side up to plastic or glass Petri dish lid containing 1 ml of lysis solution. Microwave in glass petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown. Caution: Microwave time should be monitored closely when a new or different microwave oven is used as filters can burn with flames if overdone. Microwave maximum of 6 filters at one time.

ii. Neutralize filters 5 min. on shaker at room temperature in a round vessel with ammonium acetate (4ml/filter).

iii. Briefly rinse #541 Whatman filter 2 times in 1× SSC buffer (10 ml/filter). Up to 10 filters can be combined in one container. (Filters can be dried and stored at this point.)

iv. Proteinase K treatment
Incubate up to 30 filters in proteinase K solution (10 ml/filter) for 30 min at 42°C with shaking (50 rpm) in plastic container to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

v. Rinse filter 3 times in 1× SSC (10 ml/filter) for 10 min at room temperature with shaking, 50 rpm. (Filters can be dried and stored at this point.)

4. Hybridization

i. In a plastic bag, presoak filter in hybridization buffer for 30 min at 54°C (55°C for *V. vulnificus*) with shaking, 50 rpm. Use maximum of 5 filters/bag with 10-15 ml of buffer.

ii. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/filter. Add probe (final conc is 0.5 pmol/ml) to bag with filters and incubate 1 hour at 54°C (55°C for *V. vulnificus*) with shaking. The temperature is critical for hybridization and washing steps.

iii. Rinse filter 2 times for 10 min each in 1× SSC - 1% SDS (for *tlh*) or 3× SSC - 1% SDS (for *tdh*) (10 ml/filter) at 54°C (55°C for *V. vulnificus*) with shaking. In plastic container, rinse filter 5 times for 5 min each in 1× SSC at RT with shaking, 100 rpm.

5. Color development

i. In petri dish, add place 20 ml of NBT/BCIP solution. Add filters (5 or less) to dish and incubate with shaking at RT, cover to omit light. Check development of positive control every 30 min; reaction is usually complete by 1-2 h.

ii. Rinse in tap water (10 ml/filter) 3 times for 10 min each to stop development. Do not expose filters to light as they will continue to develop. Count purple or brown spots, compared to a series of controls on separate filters. Store in dark or under acetate holders.

iii. Recover *tdh*$^+$ colonies by aligning developed filters with corresponding plate. Swab area of agar surface corresponding to positive signal with a sterile loop and streak a TCBS agar plate. Test 5-to-10 colonies with *tlh* and *tdh* probes to confirm pathogenic *V. parahaemolyticus*

6. Filter preparation for *V. parahaemolyticus* enumeration by digoxigenin-labeled probes (97)

i. The primer sequences for amplicon preparation of digoxigenin-labeled probes and the PCR conditions for construction are detailed below:

gene	protein encoded	location	sequence
tlh	thermolabile hemolysin (8)	L-TLH	5' aaa gcg gat tat gca gaa gca ctg 3'
tlh	thermolabile hemolysin (8)	R-TLH	5' gct act ttc tag cat ttt ctc tgc 3'
tdh	thermostable direct hemolysin (Kanagawa hemolysin) (87)	tdh-1	5' cca tct gtc cct ttt cct gcc 3'
tdh	thermostable direct hemolysin (Kanagawa hemolysin) (87)	tdh-4c	5' cca cta cca ctc tca tat gc 3'
trh	thermostable related hemolysin (8)	VPTRH-L	5' ttg gct tcg ata ttt tca gta tct 3'
trh	thermostable related hemolysin (8)	VPTRH-R	5' cat aac aaa cat atg ccc att tcc g 3'

ii. The following thermocycler conditions should be used:

PCR conditions	**tlh and *trh*** **Temperature**	**tlh and *trh*** **Time**	***tdh*** **Temperature**	***tdh*** **Time**
Hold	94°C	3 min	94°C	10 min
Denature	94°C	1 min	94°C	1 min
Anneal	60°C	1 min	58°C	1 min
Extend	72°C	2 min	72°C	1 min
Hold	72°C	3 min	72°C	10 min
Hold	8°C	indefinite	8°C	indefinite
	25 cycles	25 cycles	25 cycles	25 cycles

iii. Triplicate prelabeled membranes should be inoculated if hybridizations with the three *V. parahaemolyticus* dig-labeled probes are desired. Densities of *V. parahaemolyticus* can be determined with the *tlh* probe and the densities of the pathogenic strains can be determined using *tdh* and *trh* hybridizations. The results are reported as the respective number/g. Although the membranes are not sterile, careful handling will not interfere with analysis.

iv. Dilutions of a sample are prepared as previously described. One hundred µl volumes (0.2 g of 1:2 oyster homogenate) are directly plated to labeled nylon membranes on the surface of well dried T_1N_3 agar plates. A separate membrane is used for each of the three probes. Normally, the -1, -2, and -3 dilutions are adequate. The minimum detection level is 100/g (10/g for oyster). A sterile hockey stick is used to gently spread the inoculum over the membrane surface.

v. Incubate the T_1N_3 plates for 3 h at 35 ±2°C. With forceps, transfer the membrane to the surface of a TCBS agar plate and incubate overnight at 35 ±2° C.

vi. After incubation, estimate the number of green colonies and proceed with the hybridization steps. If no green colonies are present, no hybridization is necessary. The microtiter plate system of retention of suspect isolates can be used at this point by picking green colonies to plate wells with sterile toothpicks.

vii. The colonies on membranes are lysed by placing them colony side up on an absorbent pad containing 4 ml lysis soln. (Maas I) for 30 min at room temperature. A slight heating by microwaving for 20 sec may also be used.

viii. The membranes are then transferred with forceps to an absorbent pad containing 4 ml neutralizing soln. (Maas II) for 30 min at room temperature.

ix. Dry membranes briefly on a paper or cloth towel, then cross-link the DNA to the membrane for 3 min under UV light source, 254 nm, or in a UV cross-linker.

7. Day 1. Hybridization with dig-labeled probes & colorimetric or chemiluminescent detection. (97)

i. Warm shaker water bath to 65°C.

ii. Place membrane(s) in heat tolerant, sealable bag or plastic container with lid. Membranes can be stacked back to back with a fiberglass mesh screen spacer between each pair (59).

iii. Cover the stack with pre-hybridization soln. Incubate submerged at 65°C for 1 h.

iv. Remove membrane(s) from bag, container and gently wipe each with a lab tissue wetted with pre-hybridization soln. This step removes excess colony material. (This step is optional depending on colony size).

v. Cover membrane(s) with pre-hybridization soln and incubate at 65°C submerged for 2 h. Longer pre-hybridization times are acceptable.

vi. Boil double stranded dig-probe for 10 min.

vii. Pour off pre-hybridization soln and add probe while still hot. Hybridize submerged at 65°C overnight.

8. Day 2. Wash and detection steps

i. After hybridization, save probe soln in a heat resistant plastic tube in freezer. Probe can be stored up to one year.

ii. Remove membrane(s) to plastic tray and wash twice (in a stack) by covering in Wash soln A for 5 min at room temperature on shaker, 50-100 rpm.

iii. Wash membrane(s) twice by covering with pre-warmed Wash soln B in a bag or container at 65°C for 15 min.

iv. Prepare Genius Dig buffer 2 by adding 0.25 g powdered blocking reagent to 50 ml of Genius Dig Buffer 1, agitate vigorously, and microwave or put in 65°C bath to dissolve blocking reagent. Agitate every 10 min until dissolved, then cool to room temperature.

9. Colorimetric detection (option 1) or see option 2 below.

i. Prepare color solution by adding one NBT/BCIP tablet or 0.2 µ l NBT/BCIP stock solution dissolved in 10 ml of Dig buffer 3.

ii. Pour out antibody soln. Cover membrane with Dig buffer 1 in plastic tray with shaking for 15 min at room temperature, 50 rpm. (Use a freshly washed dish or bag, one that has not been in contact with anti-Dig)

iii. Pour off Dig buffer 1 and cover again in Dig buffer 1. Incubate 15 min at room temperature with shaking.

iv. Pour off Buffer 1 and cover in tray with Dig buffer 3 for 3 min at room temperature.

v. Add approximately 10 ml NBT/BCIP color substrate solution per 2-4 membranes. Membranes can be placed back-to-back (colony sides exposed). Incubate in bag or dish in dark at room temperature. Do not shake container while color is developing. The color precipitate starts to form within a few minutes and is usually complete after 12 h, but evident in 3-4 h.

vi. Once desired spots are detected, comparing with known control spots, wash membrane with 50 ml Dig buffer 1 for 5 min to stop reaction. Count the purple-to-brown spots and calculate the number/g of sample.

vii. The membrane can be stored, damp, in a bag after a brief rinse in Dig buffer 4 to retain color.

viii. Probe stripping
Nylon membranes can be stripped and re-probed if

desired. The specifics are outlined in the Roche Diagnostics manual (97).

10. For chemiluminescent detection (option 2).

    i. Warm CSPD reagent to room temperature.

    ii. Remove membrane(s) from bag/container, cover in dish with Dig buffer 1 for 1 min at room temperature.

    iii. In a container/bag, cover membrane(s) with Dig buffer 2 and incubate at room temperature on shaker tray for 1 h, 50 rpm. Longer blocking times are acceptable.

    iv. Drain off Dig buffer 2.
    Cover membrane(s) with Dig Buffer 2 with added anti-dig-alkaline phosphatase at 1:5000 (add 5 µl anti-dig for each 25 ml Dig buffer 2).

    v. Incubate for 30 min at room temperature on shaker tray, 50 rpm.

    vi. Wipe off acetate document holder with 95% EtOH. Place membrane on acetate, add 0.5 ml CSPD/100 sq cm of membrane. Wipe off outer surfaces of acetate, put in cassette with X-ray film. Expose (usually no longer than 1 h) and develop film as per manufacturer's specifications.

11. Multiplex PCR identification of *V. parahaemolyticus*(8)

    V. parahaemolyticus multiplex PCR analysis, an alternative confirmation step for suspect isolates.

    Prepare culture templates by growing overnight at 35 ± 2°C in TSB-2% NaCl. Centrifuge one ml of culture in a microcentrifuge tube for 3 min at 15,000 × g. Wash the pellet twice with physiological saline. Resuspend the pellet in 1.0 ml dH_20 and boil 10 min. Store template at -20°C until use. The following primer sets should be used:

Three primer sets (8)

tlh gene species specific (450 bp) [same as above].	L-TL	5' aaa gcg gat tat gca gaa gca ctg 3'
tlh gene species specific (450 bp) [same as above].	R-TL	5' gct act ttc tag cat ttt ctc tgc 3'
trh gene (500 bp) same as above.	VPTRH-L	5' ttg gct tcg ata ttt tca gta tct 3'
trh gene (500 bp) same as above.	VPTRH-R	5' cat aac aaa cat atg ccc att tcc g 3'
tdh gene (270 bp)	VPTDH-L	5' gta aag gtc tct gac ttt tgg ac 3'
tdh gene (270 bp)	VPTDH-R	5' tgg aat aga acc ttc atc ttc acc 3'

The following PCR reagents are recommended:

Reagent	**Reaction vol. (final conc are the same as above)**
dH_2O	28.2 µl
10× Buffer.$MgCl_2$	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

The following PCR conditions should be used:

PCR conditions:	**PCR conditions:**	**PCR conditions:**
denature	94°C	3 min
94°C	1 min	
anneal	60°C	1 min
extend	72°C	2 min
final extend	72°C	3 min
hold	8°C	indefinite
25 cycles	25 cycles	25 cycles

12. Agarose gel analysis of PCR products.

    Mix 10 µl PCR product with 2 µl 6× loading gel and load sample wells of 1.5 to 1.8% agarose gel containing 1 µg/ml ethidium bromide submerged in 1× TBE. Use a constant voltage of 5 to 10 V/cm. Illuminate gel with a UV transluminator and visualize bands relative to molecular weight marker migration. Polaroid photographs can be taken of the gel for documentation. Positive and negative culture controls and reagent control should be included with each PCR run.

V. vulnificus

Identification and Enumeration Method
Two analytical schemes for isolating and enumerating *V. vulnificus* are described. The first is a most probable number (MPN) analysis coupled with identification of suspect isolates using biochemical profiles, DNA probe colony hybridization or PCR. The second scheme includes two direct plating methods employing hybridization with DNA probes for colony identification that have been used in several studies and have been shown to be equivalent to the MPN method (29,134). A gas chromatographic technique that compares fatty acid profiles has also been successful for identifying *V. vulnificus* (68).

Seafood samples

A. Enrichment, isolation, and enumeration

    1. Prepare an initial 1:10 dilution of sample in PBS following the procedure for *V. parahaemolyticus* . Prepare decimal dilutions in PBS. Prepare a 1:10 dilution of the oyster homogenate as follows: Weigh 20 grams of the homogenate into a sterile bottle and add PBS to dilute to a final weight of 100 g. Mix by shaking. Additional 10-fold dilutions can be prepared volumetrically (i.e. 1ml of 1:10 to 9.0ml of PBS for a 1:100 dilution).

    2. Inoculate 3 × 1 ml portions of the dilutions into 3 tubes containing 10 ml APW. If low numbers are expected 2-g portions (1 g of oyster) directly from the blender can be inoculated into 3 × 100 ml APW. Incubate tubes 18 to 24 h at 35±2°C.

    3. Streak a 3 mm loopful from the top 1 cm of APW tubes with growth onto mCPC or CC selective agars. Incubate mCPC and CC agars overnight at 39-40°C (35-37°C if 39-40°C not available). On either agar, colonies are round, flat, opaque, yellow, and 1 to 2 mm in diameter.

    4. Upon identification of *V. vulnificus*, refer to the original positive dilutions of APW and apply the 3-tube-MPN tables (Appendix 2) for final enumeration of the organism.

B. Biochemical identification of isolates.

    Unless otherwise specified, all media in this section are prepared to contain a minimum of 0.5% NaCl. Note: if DNA probe or PCR is used for confirmation, steps 5-7 are not needed.

    1. Transfer two or more suspicious colonies with a needle from CC or mCPC agar plates to TSA with 2% NaCl and streak for isolation.

    2. Inoculate biochemical media using a single colony. Screening reactions, AGS, oxidase reaction, motility, and salt tolerance, are used as for *V. parahaemolyticus*..

    3. API 20E diagnostic strips can also be used. Prepare culture suspension in 2% NaCl soln. Biochemical reactions to differentiate *V. vulnificus* from *V. parahaemolyticus* can be found in Table 1.

C. DNA probe identification of species specific cytolysin gene, *vvhA* (29,134)

The oligonucleotide sequence for alkaline phosphatase label:

5'; Xga gct gtc acg gca gtt gga acc a 3';

Source of this probe is the same as for *V. parahaemolyticus*,

1. Sample preparation and dilutions are same as with MPN procedure and that presented for the AP-probe hybridization for *V. parahaemolyticus* except dry *Vibrio vulnificus* agar (VVA) is the plating medium.

2. Weigh 0.20 g of oyster:PBS homogenate directly from blender (0.1 g of oyster tissue and -1 dilution) onto VVA plate using balance with 0.01g sensitivity to tare plate.

3. Pipet 100 µl of -1 and -2 dilutions onto labeled VVA plates. From December through March plating the -1 and -2 dilutions is adequate and from May through October, summer months, -1, -2, and -3 dilutions are adequate.

4. Use sterile hockey sticks to spread oyster inoculum evenly onto VVA plates. Dry plates and uniform distribution of inoculum are essential for adequate colony isolation. Incubate plates 18-24 h at 35 ±2°C. Relatively large (1-2 mm) yellow opaque colonies (fried egg appearance) are typical of *V. vulnificus* on VVA.

5. Spotting control cultures will aid colony counting to select plates for colony lifts and to select isolates for identification and storage.

6. Plates with 25-250 typical colonies should be used for colony lifts and isolate selection if available. Additional dilutions can be used if uncertain. Colony lifts from plates with confluent growth or no typical colonies will probably be unproductive.

7. Whatman #541 filters of colony lifts are lysed and neutralized as described previously.
The microtiter plate system for multiple culture retention can be used at this point.

8. Filter preparation and Proteinase K treatment: follow the procedures outlined in section for *V. parahaemolyticus.*

D. Enumeration of *V. vulnificus* by DNA gene probe.

1. The hybridization steps are the same as for *V. parahaemolyticus*, except the temperature for hybridization and washing of filters is 55°C. All other steps including colorimetric detection are the same.

E. Confirmation of *V. vulnificus* by polymerase chain reaction (41).

1. Isolates obtained by the MPN procedure can be confirmed by PCR.

2. Primers for PCR *vvhA* or dig-labeling of probe for enumeration (519 base amplicon) are from base 785 to 1303 of the cytolysin gene. The following primers should be used:

Vvh-785F	5' ccg cgg tac agg ttg gcg ca 3'
Vvh-1303R	5'cgc cac cca ctt tcg ggc c 3'

3. The follow reagents are recommended:

Reagent	**Reaction volume (final concentrations are the same as above)**
dH_2O	28.2 µl
10× Buffer. $MgCl_2$	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

4. The following PCR conditions should be used:

PCR conditions:	**PCR conditions:**
denature	94°C 10 min
denature	94°C 1 min
anneal	62°C 1 min
extend	72°C 1 min
final extend	72°C 10 min
hold	8°C indefinite
25 cycles	25 cycles

5. Agarose gel analysis of PCR products. Mix 10 µl PCR product with 2 µl 6× loading gel and load sample wells of 1.5 to 1.8% agarose gel containing 1 µg/ml ethidium bromide submerged in 1× TBE. Use a constant voltage of 5 to 10 V/cm. Illuminate gel with a UV transluminator and visualize bands relative to molecular weight marker migration. Polaroid photographs can be taken of the gel for documentation. Positive and negative culture controls and reagent control should be included with each PCR run.

6. Culture templates are prepared by growing overnight at 35 ±2°C in TSB-2% NaCl. One ml of culture is centrifuged 15,000 × g for 3 min. The pellet is washed twice with physiological saline. The pellet is resuspended in 1.0 ml dH_20 and boiled 10 min. Template can be stored at −20°C until used.

7. The gene probe enumeration of *V. vulnificus* using dig-*vvh* follows the same direct plating procedure outlined for *V. parahaemolyticus,* except VVA is used. The hybridization and wash temperature are the same, 65°C.

Interpretation of Microbiological Findings

1. Contamination of food or water with enterotoxigenic *V. cholerae*, or *V. mimicus* (although rarely encountered) constitutes an important finding from the standpoint of public health. The entire lot of contaminated food should be withheld from distribution until the appropriate health authorities are notified and an epidemiologic investigation can be undertaken. The serogroup, biotype and enterotoxigenicity results should be reported for each sample.

2. The isolation of *V. parahaemolyticus* from seafood is not unusual. *V. parahaemolyticus* is a normal saprophytic inhabitant of the coastal marine environment and multiplies during the warm summer months (27,52). During this period the organism is readily recovered from most of the seafood species harvested in coastal areas. The virulent strains are separated from the avirulent strains of *V. parahaemolyticus* by means of the Kanagawa test or *tdh* gene detection (120). In most instances, *V. parahaemolyticus* Kanagawa-negative seafood strains do not cause human gastroenteritis. The presence of Kanagawa positive strains, or *tdh* and/or *trh* containing strains, constitutes a public health concern. A heat-processed product should not contain viable *V. parahaemolyticus* and if so, would indicate a significant problem in manufacturing practices or post-process contamination.

3. During the summer months, Gulf Coast and Mid-Atlantic shellfish normally will contain *V. vulnificus* and high levels have been isolated from warm estuarine areas (118). The organism is rare in shellfish from the West Coast. Most strains isolated have been demonstrated to be potentially virulent. Clinical, environmental and food isolates have been found to be highly virulent to mice (60,90,113,124), but infections are relatively rare even among those of increased risk (liver disease). However, those individuals at risk should be cautioned to not consume raw shellfish during certain periods of the year when levels of *V. vulnificus* are increased, normally May through October (84,104). As with *V. parahaemolyticus*, a heat processed product should not contain viable *V. vulnificus* and its isolation is a significant finding.

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