Detection, Isolation, and Identification of *Vibrio cholerae* from the Environment

Until the late 1970s and early 1980s, *Vibrio cholerae*, the causative agent of cholera, was viewed as host adapted, i.e., resident in the human gut. Numerous studies conducted during the last three decades, however, have provided clear evidence establishing the microorganism as an autochthonous (i.e., native) member of the aquatic environment (Colwell and Spira, 1992). These organisms can be detected in marine, estuarine, and fresh-water environments throughout the year if appropriately sensitive methods are employed (Colwell et al., 1981; Huq et al., 1990; Louis et al., 2000, 2003). However, *V. cholerae* may not be detected for several reasons—e.g., if it is present in low numbers, if it exists in a state that does not facilitate growth, or if it is simply overlooked. When the bacteria are viable (determined by direct-detection methods) but do not grow on conventional culture media or in broth, they are termed viable but not culturable (VBNC; Xu et al., 1982; Roszak and Colwell, 1987). *V. cholerae*, along with a host of other, mostly Gram-negative bacteria, often occur as VBNC cells in the environment, an indication that this state may be an important strategy for persistence and survival of non-spore-forming microorganisms (Colwell et al., 1985; Roszak and Colwell, 1987).

Currently, many molecular-biological tools are used to study bacteria, including VBNC cells, in clinical, environmental, or industrial samples (Knight, 2000). With the availability of these tools and a renewed interest in *V. cholerae*, the presence of these bacteria in various geographical locations has been reported with increasing frequency. However, standard methods for detection and enumeration have not been universally established. The choice of method for detection and enumeration of *V. cholerae* is largely dependent on the type of sample and the desired end purpose—i.e., simple detection or isolation. Often, combinations of different methods are used for a specific type of sample. Therefore, commonly used bacteriological, molecular, and immunological methods targeted at microbiological analysis of environmental water samples will be discussed in this unit.

Several methods will be described, and investigators will have to determine the most appropriate method consistent with their purpose and needs and with the availability of resources. For the readers’ convenience, Table 6A.5.1 provides an overview of the methods provided in this unit.

**CAUTION**: *V. cholerae* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See UNIT 1A.1 and other pertinent resources (APPENDIX 1B) for more information.

**STRATEGIC PLANNING**

There are numerous methods and protocols available in the literature for detecting and isolating *V. cholerae*. A number of them are described in this unit; however, all may not be required in one study. In addition, availability of resources in a particular laboratory will be a critical predetermining factor in making decisions on the choice of methods to include; therefore, stepwise selection cannot be standardized. In other words, it is extremely important to contrive a strategic plan to determine the choice of methods at the beginning of the study. A general method for specimen collection and transportation is provided (Basic Protocol 1). The traditional culture method (Basic Protocol 2) is the so-called “gold standard” in bacteriology, especially when an isolate is needed for the study. In the conventional culture method, selective plates are often used to reduce the
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volume of work and to identify a specific organism or variety of organisms. Once this culture method has been used and an organism isolated, a battery of biochemical tests is employed for characterization and identification. If such colonies are picked from selective plates, the number of biochemical tests needed may decline. Therefore, the number of tests may vary depending on the source of the strain. However, as more tests are performed, more convincing and accurate results will be obtained. Serogroup determination for *V. cholerae* is described in Basic Protocol 3.

Although conventional culture methods, including biochemical tests, are routinely used, they can be time consuming as well as labor intensive. The authors have included two abbreviated alternative methods that use fewer tests for a rapid presumptive identification. Colony blot hybridization (Basic Protocol 6 and Alternate Protocol 2; probe labeling is described in Support Protocol) can be used for rapid detection of *Vibrio cholerae*, and in cases involving large numbers of samples, this method may be preferred. However, it too is dependent on the culturability of the cells.

The viable but nonculturable state represents an important phenomenon, as bacteria present in this state do not grow on conventional culture media. To demonstrate the presence of the organisms in this state, a direct-detection method should be employed, notably fluorescent antibody coupled with direct viable count (DFA-DVC; Basic Protocol 7). No subsequent bacterial isolation is possible using this method, unless a parallel sample is taken. An indirect fluorescent antibody method for detecting *V. cholerae* in environmental samples is described in Basic Protocol 8.
Molecular approaches target specific genes that are unique to the organism in question. Usually, species-specific genes are targeted. This approach is extremely valuable for monitoring the presence of the organism. A single probe, designed carefully, is very specific and will be amenable to screening a larger number of strains at one time. Likewise, a series of such probes can be used for extensive characterization of an organism. These methods can be based on both culturable and nonculturable cells. Community DNA can be used to determine the presence of an organism without culture; however, results will be dependent on the quality and quantity of the extracted nucleic acids.

Strategically, if resources are available, it is appropriate to start with the conventional culture method and the DFA-DVC method. Those samples exhibiting positive results by the DFA-DVC method can be confirmed by PCR (Basic Protocol 4). If one must avoid conventional culture, one may wish to use direct PCR (Basic Protocol 5 or Alternate Protocol 1) and DFA-DVC (Basic Protocol 7), which should provide fairly convincing results. Use of PCR alone for environmental samples may not be reliable, as background noise often poses a significant obstacle in the interpretation of results.

**ISOLATION AND IDENTIFICATION OF *V. CHOLERAE* USING TRADITIONAL METHODS**

Traditional methods for isolating and identifying *V. cholerae* from environmental water samples were introduced by Colwell et al. (1977), who modified protocols originally intended for clinical samples. Briefly, concentrated water, homogenized plankton samples, or both are added to an enrichment broth and incubated overnight. The alkaline nature of the enrichment broth allows differential multiplication of *Vibrio* species.

Following enrichment, aliquots are subcultured onto selective medium—thiosulfate citrate bile salts sucrose (TCBS) agar, tellurite taurocholate gelatin agar (TTGA), or both (see Reagents and Solutions for compositions)—which offer some selective advantage to *Vibrio* species and aid in the presumptive identification of *V. cholerae*. The identity of presumptive colonies is confirmed by specific biochemical tests designed to differentiate *V. cholerae* from other species and other members of the *Vibrionaceae* and *Enterobacteriaceae*. Confirmed *V. cholerae* strains are then serogrouped as O1 or O139 by simple slide agglutination using specific antisera. Strains of *V. cholerae* that do not agglutinate with specific O1 and O139 antisera are designated as non-O1/non-O139. Stock cultures of confirmed *V. cholerae* are maintained in nutrient agar containing 0.5% NaCl overlaid with oil, or as glycerol stocks at −70°C for future analysis and reference.

**NOTE:** All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

**Specimen Collection and Transportation**

For isolation and detection of *V. cholerae* from the environment, the screening of concentrated water samples and plankton samples is recommended, since a combination of both specimen types provides a higher probability of *V. cholerae* detection (Huq et al., 1990; Binsztein et al., 2004; Huq et al., 2005). Clean, sterile, plastic sampling bottles (for water) and glass jars (for plankton) should be prepared and used. Sufficient sample (depending upon the parameters that need to be analyzed) should be collected in order to perform all examinations. Plankton samples are collected and concentrated using a 64-µm simple plankton net (Fig. 6A.5.1), preferably by towing (just below the surface, to a depth no more than 0.5 m); alternatively, this may be accomplished by manually pouring or mechanically pumping water into the net (Huq et al., 2005).
Processing of samples should begin soon after collection (“on site” or in situ) or within 1 hr of collection. If processing is delayed, the sample should be stored in a cool box at a temperature of 10° to 15°C until processing begins (not to exceed 8 hr; Clesceri et al., 1998). This is a critical parameter if enumeration of *V. cholerae* from the sample will be performed. A recent study suggests that transporting samples at ambient air temperature prior to processing can increase the number of *V. cholerae* strains isolated (Alam et al., 2006). Based on the type of examination, samples may require treatment, e.g., addition of direct viable count (DVC) reagents, before proceeding with further examination and testing. It is recommended that some basic physiochemical parameters—e.g., temperature, salinity, pH, dissolved oxygen, conductivity—of the water source be measured at the time of collection, for later analysis. Results of environmental studies conducted in Bangladesh have revealed a direct correlation of water temperature with clinical outbreaks of cholera. Furthermore, significant correlation of water depth, rainfall, conductivity, and copepod density with cholera outbreaks has been found, with lag periods from 0 to 8 weeks from optimum environmental conditions to cholera outbreaks (Huq et al., 2005). These parameters can be measured on site using portable meters.

**Materials**

- 500-ml plastic containers with caps (Nalgene) for water sampling, sterile
- Portable meter(s) that measure temperature, dissolved oxygen and pH (dissolved oxygen and pH meter Model 210A from Orion Laboratories), and salinity (Model CO150 conductivity meter from Hach Chemical Company)
- Simple plankton net, 64-µm mesh size, or nets of different mesh sizes for size filtration of plankton (Aquatic Research Instruments, [http://www.aquaticresearch.com](http://www.aquaticresearch.com); Fig. 6A.5.1)
- Net-mounted flow meter (General Oceanics; [http://www.generaloeceans.com/](http://www.generaloeceans.com/))
- Bucket of known volume (e.g., 5 liters; optional)
- 240-ml glass containers for plankton collection (Qorpak), sterile

**To collect water sample**

1a. Measure temperature, dissolved oxygen, pH, and salinity using portable meters.

2a. Uncap presterilized 500-ml plastic bottle and submerge to fill.

3a. Remove sample bottle from water. Cap bottle, leaving enough air inside for agitation and mixing.

4a. Transport samples to the laboratory for processing in a cold box (Styrofoam box with ice, 10° to 15°C), or, preferably, begin processing on site within 1 hr of collection (Basic Protocol 2).
A recent study (Alam et al., 2006) found that transportation or maintenance at ambient air temperature (22° to 25°C) after collection for up to 24 hr may enhance recovery of Vibrio species. This can be a useful alternative to increasing sample volume for geographical areas and/or seasons when the density of V. cholerae is low. This aspect of the protocol may need to be optimized for the water source and environmental conditions.

To collect plankton
1b. Rinse plankton net and cod-end of the net in the body of water to be sampled.
2b. Filter 10 to 40 liters water through the plankton net by towing; use a calibrated, net-mounted flow meter to determine volume. Alternatively, pour known volumes through the net with a small bucket.
3b. Remove cod-end from plankton net and decrease volume to 100 ml by continuing to filter.
4b. Measure (with graduated cylinder) and decant plankton sample into sterile 240-ml glass container.
5b. Transport samples to the laboratory in a cold box (Styrofoam box with ice, 10° to 15°C) for processing or, preferably, begin processing on site within 1 hr of collection (Basic Protocol 2).

See annotation to step 4a for additional considerations.

Conventional Bacteriological Culture Method
Conventional culture methods for isolating V. cholerae from environmental water samples rely on an enrichment step(s) in broth and plating on selective media, followed by confirmation using a battery of biochemical and serological tests. As stated previously, some knowledge regarding the expected prevalence of V. cholerae in the water body to be examined is useful when determining the volume of water to collect for examination. V. cholerae is an autochthonous member of the estuarine and aquatic community; however, geographic location and environmental factors such as water temperature play a critical role in determining the number of culturable V. cholerae cells present. Most water and plankton samples require concentration, with historical knowledge of the water source helping to determine the concentration factor. Water samples should be concentrated by filtration using 0.2-µm polycarbonate membrane filters (see step 1a, below). A good starting volume is 500 ml.

Overnight enrichment is performed using alkaline peptone water (APW), pH 8.6. Some investigators recommend two successive enrichments. Surface aliquots are streaked onto selective bacteriological media. The two favored selective media used for V. cholerae isolation are thiosulfate citrate bile salts sucrose (TCBS) agar and tellurite taurocholate gelatin agar (TTGA), also known as Monsur medium (Monsur, 1961). V. cholerae produces translucent, flat, yellow sucrose-fermenting colonies with elevated centers on TCBS (Fig. 6A.5.2A). The organism produces colorless colonies on TTGA (Fig. 6A.5.2B), often with a characteristic dark center after 2 days growth, surrounded by a halo, which appears due to the hydrolysis of gelatin. Both media have advantages as well as disadvantages.

TCBS, or “cholera medium,” is highly selective, inhibiting growth of Gram-positive and typical intestinal flora, which cannot grow under alkaline conditions. However, TCBS can inhibit some members of the genus Vibrio, including V. cholerae itself, as evidenced by comparing simultaneous growth of V. cholerae on TCBS versus nonselective media. In addition, TCBS can support the growth of several closely related bacteria, most notably Aeromonas and Proteus. One of the major advantages of this medium is that it

BASIC
PROTOCOL 2

Nonenteric Gamma Proteobacteria
6A.5.5
is commercially available and does not require an autoclave to prepare plates, thereby making it possible to use the medium under field conditions, without a laboratory or autoclave. TTGA medium is generally considered less selective and less “toxic” to V. cholerae than TCBS, so recovery of V. cholerae colonies may be enhanced as compared to that obtained with TCBS; however, these plates preferably should be incubated longer (48 hr versus 24 hr for TCBS) to observe the characteristic dark center. Also, the formulation is not commercially available and the medium must be autoclaved after preparation. Once presumptive strains are purified on a nonselective medium such as gelatin agar or modified nutrient agar, they are identified and confirmed by biochemical tests and serogrouped using simple slide agglutination with polyclonal V. cholerae O1 and monoclonal O139 antiserum.

**Materials**

- Plankton or water sample (Basic Protocol 1)
- Enrichment flask: 150-ml Erlenmeyer flask containing 25 ml sterile alkaline peptone water (APW), pH 8.6 (see recipe); store up to 1 month at 4°C
- Thiosulfate citrate bile-salts sucrose (TCBS) agar plates (see recipe)
- Tellurite taurocholate gelatin agar (TTGA) plates (see recipe)
- Gelatin agar (GA) plates (see recipe)
- Modified nutrient agar plates (see recipe)
- Filter paper (Whatman 3MM or equivalent) saturated with oxidase reagent (see recipe)
- 5-ml tubes of medium for biochemical tests (also see Table 6A.5.2):
  - Methyl red/Voges-Proskauer (MR-VP) broth (see recipe)
  - LB medium (**APPENDIX 4A**) containing 1% (w/v) L-arginine (pH 6.8) and 1 to 2 drops of 10% (w/v) phenol red
  - Moeller decarboxylase broth base (see recipe) containing 1% L-lysine
  - Moeller decarboxylase broth base (see recipe) containing 1% L-ornithine
  - Purple broth base (see recipe) containing 1% (w/v) arabinose
  - Purple broth base (see recipe) containing 1% (w/v) mannitol
  - Heart infusion agar (BD Biosciences) containing 0.1% (w/v) esculin and 0.05% (w/v) ferric chloride
  - Nutrient broth (BD Biosciences) with added NaCl (0%, 6%, and 8%)
  - Mineral oil, sterile
  - Kligler iron agar (KIA) slants (see recipe)
Hand-held glass tissue homogenizer, Potter-Elvehjem or Tenbroek style (Kontes), to homogenize plankton
Glass wool
Glass funnel
Filter apparatus with vacuum source
Filter membranes, 47-mm diameter, 0.22-µm pore size
30°C and 37°C incubators
Platinum inoculating loops and needles
Sterile toothpicks
Additional reagents and equipment for serogroup determination (Basic Protocol 3)

**Enrich organisms in APW**

1a. *For plankton samples:* Homogenize plankton samples using a hand-held glass tissue grinder by moving the pestle up and down in the tube, while rotating, 10 to 20 times. Add 1 ml homogenized plankton to an enrichment flask.

1b. *For water samples:* Filter 500 ml (or 100 to 1000 ml depending on bacterial density) of water first by gravity through glass wool and then under vacuum through a 47-mm, 0.22-µm pore size polycarbonate filter. Add the filter(s) with attached bacteria to an enrichment flask. Shake vigorously to detach bacteria from filter. *Large volumes may require more than one polycarbonate filter, as they will clog.*

2. Incubate the flasks statically 16 hr (overnight) at 30°C. *The flasks should not be disturbed or agitated during or after incubation, as Vibrio species tend to migrate to the liquid-air interface.*

**Selectively plate for Vibrio cholerae**

3. After enrichment, collect surface growth (which may be present as a whitish film) from the enrichment flask with a platinum inoculating loop and streak onto TCBS plates, TTGA plates, or both (i.e., selective plates).

4. Incubate the plates at 37°C 16 to 24 hr for TCBS or 48 hr for TTGA.

5. Subculture smooth, flat, sucrose-fermenting (yellow) colonies from TCBS (Fig. 6A.5.2A) and/or translucent, dark-centered colonies with halo zones from TTGA (see Fig. 6A.5.2B) onto GA and/or modified nutrient agar plates (i.e., nonselective plates). Continue incubation for 16 to 24 hr at 37°C. *When subculturing colonies from selective medium onto nonselective medium, touch the top center of the colony using an inoculating loop without touching the agar surface, to avoid carryover of contaminating growth-suppressed bacteria that may be present.*

**Characterize suspected colonies**

Suspected colonies (i.e., those selected for subculture) can be characterized using a traditional battery of biochemical tests (steps 6a to 9a), an alternate KIA-oxidase-antisera protocol (steps 6b and 7b), or a simple two-test protocol (step 6c to 8c).

To characterize by traditional battery of biochemical tests and serogroup determination

6a. Pick suspected colonies from GA and/or modified nutrient agar plates using sterile toothpicks or a platinum loop. Streak onto filter paper saturated with oxidase reagent. *V. cholerae is oxidase-positive, as indicated by the rapid appearance (10 to 30 sec) of a purple color. A delayed color change (60 sec or longer) or no color change is indicative of a negative result.*
<table>
<thead>
<tr>
<th>Test</th>
<th>Medium</th>
<th>Temperature</th>
<th>Duration</th>
<th>Reagent to add prior to reading</th>
<th>Positive result indicates</th>
</tr>
</thead>
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<tr>
<td>Methyl Red</td>
<td>MR-VP medium</td>
<td>37°C</td>
<td>48 hr</td>
<td>5-6 drops methyl red indicator&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ability to produce stable acid end products from glucose fermentation</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>MR-VP medium</td>
<td>37°C</td>
<td>48 hr</td>
<td>3 ml Barritt’s reagent A&lt;sup&gt;b&lt;/sup&gt; and 1 ml Barritt’s reagent B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Production of acetoin, formed from pyruvic acid during glucose fermentation</td>
</tr>
<tr>
<td>Arginine dihydrolase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>LB medium (&lt;i&gt;APPENDIX 4A&lt;/i&gt;) containing 1% (w/v) L-arginine (pH 6.8) and 1-2 drops of 10% (w/v) phenol red</td>
<td>37°C</td>
<td>24 hr</td>
<td>None</td>
<td>Presence of the enzyme arginine dihydrolase</td>
</tr>
<tr>
<td>Lysine decarboxylase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Moeller decarboxylase broth base (see recipe) containing 1% (w/v) L-lysine</td>
<td>37°C</td>
<td>24 hr</td>
<td>None</td>
<td>Presence of the enzyme lysine decarboxylase</td>
</tr>
<tr>
<td>Ornithine decarboxylase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Moeller decarboxylase broth base (see recipe) containing 1% (w/v) L-ornithine</td>
<td>37°C</td>
<td>24 hr</td>
<td>None</td>
<td>Presence of the enzyme ornithine decarboxylase</td>
</tr>
<tr>
<td>Arabinose, acid production</td>
<td>Purple broth base (see recipe) containing 1% (w/v) arabinose</td>
<td>37°C</td>
<td>24 hr</td>
<td>None</td>
<td>Utilization of the carbohydrate arabinose by production of acid end products</td>
</tr>
<tr>
<td>Mannitol, acid production</td>
<td>Purple broth base (see recipe) containing 1% (w/v) mannitol</td>
<td>37°C</td>
<td>24 hr</td>
<td>None</td>
<td>Utilization of the carbohydrate mannitol by production of acid end products</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Oxidase reagent: 1% (w/v) N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride</td>
<td>37°C</td>
<td>10-30 sec</td>
<td>None</td>
<td>Presence of cytochrome oxidase</td>
</tr>
<tr>
<td>Esclusin hydrolysis</td>
<td>Heart infusion agar containing 0.1% (w/v) esculin and 0.05% (w/v) ferric chloride</td>
<td>37°C</td>
<td>3 days</td>
<td>None</td>
<td>Hydrolysis of esculin to glucose and esculetin</td>
</tr>
<tr>
<td>Growth in nutrient broth with 0% NaCl</td>
<td>Nutrient broth&lt;sup&gt;e&lt;/sup&gt; with 0% NaCl</td>
<td>37°C</td>
<td>5 days</td>
<td>None</td>
<td>Organism has minimal salt requirement</td>
</tr>
<tr>
<td>Growth in nutrient broth with 6% NaCl</td>
<td>Nutrient broth&lt;sup&gt;e&lt;/sup&gt; with 6% (w/v) NaCl</td>
<td>37°C</td>
<td>5 days</td>
<td>None</td>
<td>Organism is tolerant of moderate salt concentration</td>
</tr>
<tr>
<td>Growth in nutrient broth with 8% NaCl</td>
<td>Nutrient broth&lt;sup&gt;e&lt;/sup&gt; with 8% (w/v) NaCl</td>
<td>37°C</td>
<td>5 days</td>
<td>None</td>
<td>Organism is tolerant of high salt concentration</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dissolve 0.1 g methyl red in 300 ml of 95% ethanol. Adjust volume to 500 ml with water. Store up to 6 months at 2° to 8°C.

<sup>b</sup>Dissolve 5 g α-naphthol in 100 ml absolute ethanol. Store up to 6 months at 2° to 8°C.

<sup>c</sup>Dissolve 40 g potassium hydroxide in 100 ml distilled water. Store up to 6 months at 2° to 8°C.

<sup>d</sup>For lysine and ornithine decarboxylase, it is useful to inoculate a tube of decarboxylase broth base without the addition of either amino acid for comparison as a negative control. All decarboxylase test tubes must be overlaid with mineral oil.

<sup>e</sup>Nutrient broth must not contain any sodium chloride.
### Table 6A.5.3  Expected Results of *V. cholerae* for Selected Biochemical Tests

<table>
<thead>
<tr>
<th>Test</th>
<th>% Positive</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl Red</td>
<td>99</td>
<td>Red</td>
<td>Yellow</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>75</td>
<td>Red</td>
<td>Copper</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>0</td>
<td>Pink</td>
<td>Orange</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>99</td>
<td>Dark purple</td>
<td>Yellow</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>99</td>
<td>Dark purple</td>
<td>Yellow</td>
</tr>
<tr>
<td>Arabinose, acid production</td>
<td>0</td>
<td>Yellow</td>
<td>Purple</td>
</tr>
<tr>
<td>Mannitol, acid production</td>
<td>99</td>
<td>Yellow</td>
<td>Purple</td>
</tr>
<tr>
<td>Oxidase</td>
<td>100</td>
<td>Purple</td>
<td>Clear</td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>0</td>
<td>No fluorescence&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>Growth in nutrient broth with 0% NaCl</td>
<td>100</td>
<td>Turbid</td>
<td>Clear</td>
</tr>
<tr>
<td>Growth in nutrient broth with 6% NaCl</td>
<td>53</td>
<td>Turbid</td>
<td>Clear</td>
</tr>
<tr>
<td>Growth in nutrient broth with 8% NaCl</td>
<td>1</td>
<td>Turbid</td>
<td>Clear</td>
</tr>
</tbody>
</table>

<sup>a</sup>Traditional biochemical characterization of presumptive colonies (also see Table 6A.5.2). Adapted from (Lennette et al., 1985).

<sup>b</sup>Loss of fluorescence is used since many *V. cholerae* strains produce melanin, which interferes with the normal indicator (darkening of butt).

7a. If a positive result is achieved using the oxidase reagent test, inoculate one tube of each of the other tests outlined in Table 6A.5.2 (eleven total after oxidase test has been performed) for each suspected colony. Inoculate a second tube of each test with a known positive control. Include a tube of decarboxylase base medium (without the amino acid supplement) as a negative control for the lysine and ornithine decarboxylase tests. Refer to Table 6A.5.3 for interpretation of results.

8a. Overlay arginine, lysine, and ornithine tubes (and decarboxylase broth base used as negative control) with sterile mineral oil. Incubate all tubes at 37°C for the duration indicated in Table 6A.5.2.

9a. Proceed to serogroup determination (Basic Protocol 3) with presumptive *V. cholerae* colonies.

**Characterize colonies by simple, rapid biochemical procedure and serogroup determination**

6b. Assay suspected colonies from GA and/or modified nutrient agar (from step 5) for arginine dihydrolase and esculin hydrolysis as described in Tables 6A.5.2 and 6A.5.3.

7b. Proceed to serogroup determination (Basic Protocol 3) with presumptive *V. cholerae* colonies (negative for arginine dihydrolase and negative for esculin hydrolysis).

**Characterize colonies by KIA-oxidase and serogroup determination**

6c. Stab-inoculate suspected colonies from GA and/or modified nutrient agar into KIA slants using an inoculating needle. Incubate at 30°C overnight.

*Stab inoculation is accomplished by passing an inoculating needle that has been touched to a suspected colony on the GA or modified nutrient agar plate vertically into the surface of the agar at the center of the slant and down completely into the tube.*

*See WHO (1974) and Tison (1999) as well as the Commentary in this unit, for more detail.*
Detection, Isolation, and Identification of \textit{V. cholerae} from the Environment

6A.5.10

7c. Perform the oxidase test (step 6a) on colonies giving an alkaline slant over acid butt (purple slant and yellow butt) with no gas production or blackening of the butt due to H$_2$S production.

8c. Proceed to serogroup determination (Basic Protocol 3) with presumptive \textit{V. cholerae} colonies.

**BASIC PROTOCOL 3**

**Serogroup Determination**

Over 210 serogroups of \textit{V. cholerae} have been described to date based on antigenic properties of cell surface polysaccharides, of which serogroup O1 and O139 have been implicated in epidemics of cholera, while serogroup O37 has been held responsible for localized outbreaks of cholera in Czechoslovakia and Sudan. The remaining serogroups collectively and commonly termed “non-O1/non-O139” are predominant among the strains of \textit{V. cholerae} isolated from the aquatic environment (Sack et al., 2003). Although reported mostly in O1 and O139 serogroups, the cholera toxin gene has been reported in non-O1/non-O139 strains, also from the aquatic environment, in India (Chakraborty et al., 2000). However, because of the epidemic potential, the method to determine O1 and O139 serogroup is described below. Strains other than O1 or O139 serogroup need not be serogrouped unless there is a special need, in which case those strains should be sent to a Reference Center for serotyping, since antisera for serogroups other than O1 and O139 are not commercially available.

**Materials**

- Phosphate buffered saline (PBS; \textit{APPENDIX 2A})
- \textit{V. cholerae} colonies (6- to 16-hr subculture on nonselective modified nutrient agar, e.g., Basic Protocol 2)
- Polyvalent antisera for serogroup O1 and O139 \textit{V. cholerae} (BD Biosciences)
- Glass microscope slides

1. Add two separate drops of PBS to a microscope slide.
2. Add a loopful of fresh growth from a 6 to 16 hr subculture of \textit{V. cholerae} on nonselective medium to each drop, and mix.
3. Add an equal-sized drop of group O1 polyvalent antiserum to one of the drops.
4. Mix the antiserum-culture suspension by tilting the slide back and forth. Determine if the reaction clumps (i.e., agglutinates) within 0.5 to 1 min, indicating a positive result.

Autoagglutination, i.e., clumping in the saline solution without antiserum, is indicative of a “rough” morphotype which cannot be typed by antisera. For typing of colonies with rough morphotype, see Alternate Protocol 1.

5. Test non-O1 serogroup colonies using O139 antisera, repeating steps 1 to 4, but substituting anti-O139 antiserum for anti-O1 antiserum.

**MOLECULAR METHODS OF DETECTION AND IDENTIFICATION OF \textit{V. CHOLERAE}**

Following the introduction of molecular methods into bacteriology, there has been an influx of newer techniques aimed at understanding the mechanism of infection and at the detection of pathogens, or organisms with virulent properties, in clinical or environmental samples. Soon after the \textit{V. cholerae} genome sequence was published in 2000 (Heidelberg et al., 2000), PCR-based simple molecular probes became an important tool for detecting \textit{V. cholerae} and its antigens. Direct-detection methods have proven to be important when conventional culture methods are recognized as ineffective because of the viable but not culturable (VBNC) organisms present in samples collected for analysis.
Identification of Suspected or Presumptive *V. cholerae* by PCR

The polymerase chain reaction (Kramer and Coen, 2001) is a useful alternative to labor-intensive biochemical tests, which are sometimes difficult to interpret and often require replication. Also, many biochemical tests require an additional overnight or longer incubation before results are obtained. It is largely up to the investigator to determine at which step in the traditional, culture-based isolation procedure to switch to this molecular method. Ideally, at least minimal biochemical tests (Basic Protocol 2, step 6b or steps 6c and 7c) should be performed before switching to PCR identification, to decrease the number of strains to be screened to a manageable number. In this method, crude template is prepared by boiling to lyse the cells. This template is then amplified using PCR primers specific to *V. cholerae*, which target the internal transcribed spacer (ITS) region between 16S and 23S rRNA. It is convenient at this stage to characterize a confirmed *V. cholerae* strain by screening for several genes associated with pathogenesis (*ctxA*, *toxR*, *tcpA*, *zot*, and *ompU*) by PCR. PCR products are analyzed by gel electrophoresis and visualized under UV light with ethidium bromide. Positive and negative controls should be run in parallel and should include a eubacterial 16S rDNA PCR reaction on each sample to test template quality. (See Table 6A.5.4 for PCR primers, expected amplicon size and reference).

**Materials**

- Overnight culture of putative *V. cholerae* in liquid medium or on agar plates (Basic Protocol 2)
- H₂O, sterile
- 1× PCR amplification buffer (*APPENDIX 2A*) containing 15 mM MgCl₂ and 0.1% (w/v) gelatin
- 25 mM dNTP stock: dATP, dCTP, dGTP, dTTP (*APPENDIX 2A*)
- 20 µM PCR primers for ITS (Table 6A.5.4)
- Taq DNA polymerase
- 1.5% agarose gel (Voytas, 2000)
- TAE buffer (*APPENDIX 2A*)
- Molecular weight ladder (e.g., Hyperladder IV, Bioline)
- 1 µg/ml ethidium bromide (*APPENDIX 2A*)
- 2-ml microcentrifuge tubes, sterile
- Boiling water bath
- PCR tubes
- Thermal cycler (MJ Research)
- UV transilluminator, handheld UV lamp, or digital gel documentation system
- Additional reagents and equipment for agarose gel electrophoresis and ethidium bromide staining of gels (Voytas, 2000)

**Prepare crude template**

1a. From liquid medium: Centrifuge a 1-ml culture (overnight growth at 37°C) and resuspend in 1 ml sterile water.

1b. From agar plates: Resuspend a loopful of pure culture (50 to 100 colonies) of suspected or presumptive *V. cholerae* into 300 µl sterile water.

*Plates should contain a fresh overnight subculture.*

2. In a sterile 2-ml microcentrifuge tube, dilute suspension 1:1000 in sterile water to a final volume of 1 ml.

*Alternatively, a single isolated colony can be resuspended in 20 µl sterile water.*
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>pVC-F2</td>
<td>TTAAGCSTTTTTCCTGAGAATG</td>
<td>295-310</td>
<td>Chun et al., 1999</td>
</tr>
<tr>
<td></td>
<td>PVMC-R1</td>
<td>AGTCACTTAACCATACAACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxA</td>
<td>PCTA-94F</td>
<td>CGGACCAGATTCTAGACCTCTGT</td>
<td>563</td>
<td>Fields et al., 1992</td>
</tr>
<tr>
<td></td>
<td>PCTA-614R</td>
<td>CGATGATCTTGGAGCATTCCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>toxR</td>
<td>pToxR-101F</td>
<td>CTTTCGATCCCTAAGCAATAC</td>
<td>778</td>
<td>Rivera et al., 2001</td>
</tr>
<tr>
<td></td>
<td>pToxR-837R</td>
<td>AGGGTTAGCAACCGATGGTAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA</td>
<td>pTcpA-72F</td>
<td>CACGATAAGAAAACCGGTAAGAG</td>
<td>452-621</td>
<td>Keasler and Hall, 1993</td>
</tr>
<tr>
<td></td>
<td>pTcpAE77R</td>
<td>CGAAAGACCTCTTGTACGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pTcpACL-647R</td>
<td>TTACAAATGCAACGCCGGAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zot</td>
<td>PZot-225F</td>
<td>TCGCTTACAGGCGGGTCTTGT</td>
<td>946</td>
<td>Rivera et al., 2001</td>
</tr>
<tr>
<td></td>
<td>PZot-1129R</td>
<td>AACCCTGTTTCACTTACCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ompU</td>
<td>pOmpU-80F</td>
<td>ACGCTGACGGAATCAACCAAG</td>
<td>868</td>
<td>Rivera et al., 2001</td>
</tr>
<tr>
<td></td>
<td>pOmpU-906R</td>
<td>GCCGAAGTTTGGCTGTAAGTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxA</td>
<td>VCT1</td>
<td>ACAGAGTGAGTGACCTTTGACC</td>
<td>308</td>
<td>Hoshino et al., 1998</td>
</tr>
<tr>
<td></td>
<td>VCT2</td>
<td>ATACCATCCATATATTTGGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O1-rfb</td>
<td>O1F2-1</td>
<td>GTTTCTCACTGAAGAGTGGG</td>
<td>192</td>
<td>Hoshino et al., 1998</td>
</tr>
<tr>
<td></td>
<td>O1R2-2</td>
<td>GGTCACTCTGTAAGTACAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O139-rfb</td>
<td>O139F2</td>
<td>AGCCTTTTTTATACGCGGTTG</td>
<td>449</td>
<td>Nandi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>O139R2</td>
<td>GTCAACCCGATCGTAAAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ompW</td>
<td>ompW-F</td>
<td>CACCAAGAGGGTGAGCTTTATTTGTT</td>
<td>588</td>
<td>Nandi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>ompW-R</td>
<td>GAACCTTAAACCCGCGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxA</td>
<td>CtxA-F</td>
<td>CTCAGACGGGTGTTAGGCACG</td>
<td>302</td>
<td>Shirai et al., 1991; Nandi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>CtxA-R</td>
<td>TCTACTCTCTGAGCCTATATACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rDNA</td>
<td>16S-F</td>
<td>CAGCMGCCGGCGGTAAATWC</td>
<td>888</td>
<td>Amann et al., 1995</td>
</tr>
</tbody>
</table>

Currently, there are no published 23S rDNA PCR primers specific for V. cholerae.

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3. Place the microcentrifuge tube containing the resuspended culture into a boiling water bath for 10 min.

4. Cool tube to room temperature by allowing tube to sit on bench (≈30 min).

**Perform Vibrio cholerae–specific PCR (Chun et al., 1999)**

5. Set up V. cholerae–specific ITS (internal transcribed spacer region) PCR in a PCR tube for a total reaction volume of 25 µl containing the following:

- 5 µl template (from step 4)
- 1× PCR amplification buffer
- 200 µM each dNTP
- 800 nM forward and reverse primers for ITS (pVC-F2, pVCM-R1)
- 0.625 U Taq DNA polymerase.
6. Amplify the *V. cholerae*-specific ITS target with the following cycling conditions:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final</td>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Refer to Kraemer and Coen (2001) for information about optimizing PCR.

**Perform agarose gel electrophoresis and visualize results**

7. Run PCR product out on a 1.5% agarose gel in 1 x TAE buffer for 1 to 2 hr at 5 V/cm (Voytas, 2000) along with a molecular weight ladder (e.g., Hyperladder IV).

8. Stain the gel by incubating in 1 µg/ml ethidium bromide staining solution for 15 min at room temperature.

9. Destain the gel by incubating in distilled water for 15 min at room temperature.

10. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

*The V. cholerae 16S-23S rDNA intergenic spacer region amplicon is 300 bp in size (Fig 6A.5.3; lane 2).*

**Screen for toxigenic factors**

11. Screen ITS-PCR confirmed *V. cholerae* isolates for the toxigenic factors *toxR*, *tcpA*, *ctxA*, *zot*, and *ompU* by PCR according to steps 1 to 10 above, using the PCR primers pToxR, pTcpA, pCTA, pZot, and pOmpU, respectively (see Table 6A.5.4 and Fig. 6A.5.3).

![Figure 6A.5.3](image-url)  
*Figure 6A.5.3* Results (gel) of PCR assays used to detect and characterize *V. cholerae*. Lane 1, Hyperladder IV (Bioline); lane 2, *V. cholerae*-specific ITS; lane 3, *ctxA* (pCTA); lane 4, *tcpA* of *V. cholerae* O1 Classical; lane 5, *tcpA* of *V. cholerae* O1 El Tor; lane 6, *tcpA* of *V. cholerae* O139; lane 7, *toxR*; lane 8, *zot*; lane 9, *ompU*; lane 10, O1-O139/ctxA multiplex of *V. cholerae* O1 and O139. See Table 6A.5.4 for amplicon sizes.
Detection, Isolation, and Identification of V. cholerae from the Environment

**BASIC PROTOCOL 5**

**Direct PCR for Environmental Samples**

Despite the ubiquitous nature of *V. cholerae*, isolation and detection by traditional methods are difficult since these methods rely on cultivating the organism. These difficulties arise from several possible factors: low density, interspecific competition, cell state (VBNC), and cell health (e.g., starvation). The polymerase chain reaction offers a molecular-based alternative to the traditional culture and immunological methods discussed later. Three types of targets are used to detect *V. cholerae* in environmental samples by PCR: species-specific genes (16S rDNA, ITS, *ompW*); serogroup-specific genes (O1 and O139 *rbf*); and toxin and pathogenic factor genes (e.g., *ctx*, *tcpA*). Briefly, water, plankton, and/or sediment samples are collected and concentrated. DNA is extracted from the samples using a modification of the method of Murray and Thompson (1980) and PCR is performed on the extracted DNA using a multiplex (*ompW* and *ctxA*) primer array. This multiplex PCR can be substituted for the total *V. cholerae* ITS-based PCR identification described in Basic Protocol 4, if desired. Positive and negative controls should be run in parallel and should include a eubacterial 16S rDNA PCR reaction on each sample to test template quality (see Table 6A.5.4 for PCR primers and references).

**Materials**

- Water or plankton sample (Basic Protocol 1) or sediment sample
- TE buffer, pH 8.0 (*APPENDIX 2A*)
- 10% (w/v) SDS (*APPENDIX 2A*)
- 20 mg/ml (2%) proteinase K
- 5 M NaCl
- CTAB/NaCl solution (see recipe)
- 25:24:1 phenol/chloroform/isoamyl alcohol (*APPENDIX 2A*)
- 24:1 chloroform/isoamyl alcohol
- Isopropanol
- 70% ethanol
- 1× PCR amplification buffer (*APPENDIX 2A*) containing 15 mM MgCl₂ and 0.1% (w/v) gelatin
- 25 mM dNTP stock: dATP, dCTP, dGTP, dTTP (*APPENDIX 2A*)
- 20 µM forward and reverse PCR primers (Table 6A.5.4) for *ompW* (*ompW*-F, R) and *ctxA* (*ctxA*-F, -R)
- *Taq* DNA polymerase
- 1.5% agarose gel (Voytas, 2000)
- TAE buffer (*APPENDIX 2A*)
- Molecular weight ladder (e.g., Hyperladder IV, Bioline)
- 1 µg/ml ethidium bromide (*APPENDIX 2A*)
- 37° and 65°C water baths
- Vacuum desiccator or lyophilizer
- Thermal cycler (MJ Research)
- UV transilluminator, handheld UV lamp, or digital gel documentation system

Additional reagents and equipment for sample collection/transportation (Basic Protocol 1) and enrichment (Basic Protocol 2), PCR (Kramer and Coen, 2001), agarose gel electrophoresis and ethidium bromide staining of gels (Voytas, 2000), and isolation of *V. cholerae* by traditional culture method (Basic Protocol 2; optional)
Enrich environmental sample
For water and plankton samples
1a. Transport samples to the laboratory as for the conventional bacterial culture method (Basic Protocol 1).
2a. Filter (water sample) or homogenize (plankton sample), then perform enrichment in APW as in the conventional bacterial culture method (Basic Protocol 2).

For sediment samples
1b. Add sediment to 100 ml distilled water until the final volume reaches 200 ml. Mix well and allow sediment to settle. Remove large particulate matter by centrifuging a 10-ml aliquot of the slurry for 8 min at 1000 × g, room temperature.
2b. Enrich 1 ml of the sediment slurry (supernatant from previous step) in an APW enrichment flask, as for water and plankton samples (Basic Protocol 2).

Extract DNA
3. Microcentrifuge a 1-ml aliquot from the upper surface (i.e., top 1 to 2 mm) of the APW enrichment culture for 5 min at 12,000 × g, room temperature.
4. Resuspend the cell pellet in 567 µl TE buffer, pH 8.0.
5. Add 30 µl of 10% SDS, then 3 µl of 20 mg/ml proteinase K. Incubate the suspension 1 hr at 37°C.
6. Add 100 µl of 5 M NaCl, then add 80 µl of CTAB/NaCl solution. Incubate mixture 10 min at 65°C.
7. Add 800 µl of 25:24:1 phenol/chloroform/isoamyl alcohol, vortex, and centrifuge 5 min at 12,000 × g, room temperature.
8. Transfer aqueous (upper) phase to a new microcentrifuge tube. Add 800 µl of chloroform/isoamyl alcohol (24:1), vortex, and centrifuge 5 min at 12,000 × g, room temperature.
9. Transfer aqueous (upper) phase to a new microcentrifuge tube. Precipitate DNA by adding an equal volume of isopropanol.
10. Microcentrifuge 5 min at 12,000 × g, room temperature, and remove the supernatant. Wash DNA by adding 1 ml of 70% ethanol and microcentrifuging 5 min at 12,000 × g, room temperature.
11. Dry pellet in vacuum desiccator or lyophilizer and resuspend in 100 µl TE buffer, pH 8.0.

Perform multiplex PCR assay for detection of ompW (V. cholerae-specific) and ctxA (toxigenicity) (Nandi et al., 2000)
12. Set up ompW-ctxA multiplex PCR in a PCR tube for a total reaction volume of 25 µl containing the following:

10 to 20 ng extracted genomic DNA (step 11)
1 × PCR amplification buffer
250 µM each dNTP
1.2 pmol/µl forward and reverse primers for ompW (ompW-F, -R)
0.25 pmol/µl forward and reverse primers for ctxA (ctxA-F, -R)
0.625 U Taq DNA polymerase.

This protocol may be modified for the analysis of cultured organisms by using crude template DNA (see Basic Protocol 4, step 4) in this mixture instead of extracted genomic DNA.
13. Amplify the targets with the following cycling conditions:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 min</td>
<td>94°C</td>
</tr>
<tr>
<td>30</td>
<td>30 sec</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>30 sec</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>30 sec</td>
<td>72°C</td>
</tr>
<tr>
<td>1</td>
<td>7 min</td>
<td>72°C</td>
</tr>
</tbody>
</table>

(initial denaturation) (denaturation) (annealing) (extension) (final extension).

*Perform agarose gel electrophoresis and visualize results*

14. Run PCR product out on a 1.5% agarose gel in 1× TAE for 1 to 2 hr at 5 V/cm (Voytas, 2000), along with a molecular weight ladder (e.g., Hyperladder).

15. Stain the gel by incubating in 1 µg/ml ethidium bromide staining solution for 15 min at room temperature.

16. Destain the gel by incubating in distilled water for 15 min at room temperature.

17. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

*The ompW and ctxA amplicons are 588 and 302 bp in length, respectively.*

18. Screen samples giving a positive result for isolation of *V. cholerae* using the traditional culture method as described in Basic Protocol 2, if desired.

**ALTERNATE PROTOCOL 1**

*Alternative Multiplex PCR Assay for Detection of O1 and O139 Serogroup V. cholerae and ctxA*

In the following procedure (Hoshino et al., 1998), the multiplex PCR assay is performed to confirm O1 and O139 somatic antigens and for the simultaneous detection of the toxigenic trait gene, *ctxA*. This protocol can be used in place of steps 12 to 17 of the *ompW-ctxA* method (see Basic Protocol 5) for direct detection of *V. cholerae* in environmental samples. In addition, this assay can be used in place of the *V. cholerae*-specific ITS PCR (see Basic Protocol 4) for confirmation of presumptive *V. cholerae* strains isolated by the conventional bacteriological culture method. This protocol is particularly useful for determining the O1 or O139 serogroups of strains that are of the rough morphotype (see Basic Protocol 3, step 4 annotation) in which the serogroup cannot be determined by agglutination with specific antiserum.

**Additional Materials (also see Basic Protocol 4 or 5)**

- 20 µM forward and reverse PCR primers (Table 6A.5.4) for O1-rfb (O1F2-1, O1R2-2) and for O139-rfb (O139F2, O139R2)
- 2.0% agarose gel (Voytas, 2000)

1. Prepare crude template (Basic Protocol 4) or DNA extract (Basic Protocol 5).

2. Set up O1/O139-rfb/ctxA multiplex PCR in a PCR tube for a total reaction volume of 30 µl containing the following:

- 10 to 20 ng of crude DNA template (Basic Protocol 4) or extracted genomic DNA (Basic Protocol 5)
- 1× PCR amplification buffer
- 210 µM each dNTP
- 0.5 µM forward and reverse primers for O1-rfb (O1F2-1, O1R2-2)
- 0.27 µM forward and reverse primers for O139-rfb (O139F2, O139R2)
- 0.17 µM forward and reverse primers for *ctxA* (VCT1, VCT2)
- 0.75 U *Taq* DNA polymerase.
3. Amplify the targets with the following cycling conditions:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 min</td>
<td>94°C (initial denaturation)</td>
</tr>
<tr>
<td>35</td>
<td>1 min</td>
<td>94°C (denaturation)</td>
</tr>
<tr>
<td></td>
<td>1 min</td>
<td>55°C (annealing)</td>
</tr>
<tr>
<td></td>
<td>1 min</td>
<td>72°C (extension)</td>
</tr>
<tr>
<td>Final extension</td>
<td>7 min</td>
<td>72°C (final extension).</td>
</tr>
</tbody>
</table>

4. Run PCR product out on a 2.0% agarose gel in 1× TAE for 1 to 2 hr at 5 V/cm (Voytas, 2000), along with a molecular weight ladder (e.g., Hyperladder).

5. Stain the gel by incubating in 1 µg/ml ethidium bromide staining solution for 15 min at room temperature.

6. Destain the gel by incubating in distilled water for 15 min at room temperature.

7. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

   The O1-rfb, O139-rfb, and ctxA amplicons are 192, 449, and 308-bp in length, respectively (see Fig 6A.5.3).

8. Screen samples giving a positive result for isolation of *V. cholerae* using the traditional culture method as described in Basic Protocol 2, if desired.

**Colony Blot Hybridization with Labeled RNA or DNA Probes**

The colony lift procedure is used to immobilize DNA from bacterial colonies onto nitrocellulose or nylon filters to allow quick screening of a large number of colonies for genetic elements of interest by hybridization. The colony blot hybridization procedure is a culture-based alternative to the conventional culture method (Basic Protocol 2), so it is dependent upon the presence of *V. cholerae* in the sample as viable, culturable cells. Detection by hybridization precludes the necessity of numerous biochemical tests. Its advantage over PCR is that isolation is performed simultaneously with blot preparation, and enumeration can be performed more easily. Briefly, LB or modified nutrient agar spread plates are prepared from water samples and incubated overnight. Other plating media can be used, but the medium should be relatively rich and nonselective to allow for vigorous growth and cells with a high RNA content. Nitrocellulose (or nylon) membranes are overlaid, lifted, and treated to bind RNA (or DNA) to the membrane (Rehnstam et al., 1989). Plates to be lifted should contain 50 to 150 well defined colonies, 2.0 to 3.0-mm in size. Membranes should be handled with sterile forceps only and can be sterilized in an autoclave between two pieces of filter paper for 15 min prior to use. For RNA blots, care should be taken to minimize RNase contamination. Blots are then hybridized with labeled probe specific for *V. cholerae* (and *V. mimicus*), 5′-ACTTTGTGAGATTCGCTCCACCTCG-3′ (Heidelberg, 1997; Heidelberg et al., 2002), or toxigenic *V. cholerae* (ctxA). *V. mimicus* is a species closely related to *V. cholerae*, previously described as biochemically atypical *V. cholerae* (nonsucrose fermenting indicated by green colonies instead of yellow colonies on TCBS). *V. mimicus* produces a variety of toxins, including cholera toxin (potential reservoir), and has caused sporadic diarrhea (Ramamurthy et al., 1994). Fluorescently labeled probes are preferred (e.g., see Boyle and Perry-O’Keefe, 1992); however, the DIG system (Roche; see Alternate Protocol 2) offers a good alternative when a variable mode imager (such as Typhoon from GE Healthcare) or equivalent machine for detection of the fluorochrome, such as Dark Reader (Clare Chemical Research, http://www.clarechemical.com/), is not available.
Detection, Isolation, and Identification of *V. cholerae* from the Environment

6A.5.18

### Materials

- Enrichment flask with *Vibrio cholerae* culture (see Basic Protocol 2) (for detection) or unenriched sample (for enumeration)
- Alkaline peptone water (APW; see recipe)
- LB plates *(APPENDIX 4A)* or modified nutrient agar plates (see recipe)
- 10% (w/v) SDS *(APPENDIX 2A)*
- 3× SSC *(APPENDIX 2A)*
- Prewashing solution (see recipe), 60°C
- DEPC-treated H2O *(APPENDIX 2A)*
- RNA colony blot hybridization solution (see recipe)
- 25 ng/µl fluorescein-labeled Vchomim1276 probe (5′-ACTTTGTGAGATTGCTCCACCTCG-3′, with 5′ fluorescein label; Sigma Genosys)
- Washing solution for colony blot hybridization with fluorescein-labeled probes (see recipe), 60°C
- 85-mm sterile nitrocellulose membranes, 0.22-µm (GE Water and Process Technologies)
- 65°C incubator
- Pyrex dishes
- Whatman no. 3 filter paper cut slightly larger than the nitrocellulose membranes
- 70°C oven
- 60°C water bath
- Hybridization oven
- Typhoon Scanner (GE Healthcare) or Dark Reader (Clare Chemical)

**NOTE:** All solutions should be DNase and RNase-free. For RNA colony blot hybridization, use DEPC-treated water *(see APPENDIX 2A)* to make hybridization solutions.

### Prepare spread plates

1a. For detection: Prepare spread plates from enrichment flasks using APW as diluent by plating three 10-fold serial dilutions onto LB or modified nutrient agar plates.

1b. For enumeration (and detection): Prepare spread plates by plating three 10-fold serial dilutions onto LB or modified nutrient agar plates on-site without APW enrichment.

**Alternatively, 100 to 500 ml of water may be filtered through the 0.22-µm nylon membranes and overlaid onto an agar plate. If this method is preferred, incubate the plate containing the membrane overnight at 30°C and then proceed to step 6.**

2. Incubate plates overnight at 37°C.

### Perform colony lift

3. Using a pencil, mark each membrane on the top side with the blot ID (e.g., recording the medium, sample, dilution) that matches the plate to be lifted. Also place asymmetrical orientation marks on the membrane to facilitate later comparison between membrane and plate.

4. Overlay the corresponding membrane onto each of the incubated spread plates, starting from the center to ensure there are no air bubbles.

5. Allow at least 15 min for transfer.

6. Replica plate the membrane onto a fresh modified nutrient agar plate, transferring the orientation markings. Maintain master and replica plate at 10° to 15°C, sealed with Parafilm.

**Perform this step only when subsequent analysis of a positive colony is necessary.**
7. For each plate, prepare two Pyrex dishes by placing a piece of Whatman no. 3 filter paper cut slightly larger than the nitrocellulose membrane into each. Prewet the filter paper in one dish with 10% SDS and the other with 3× SSC, pouring or pipetting the liquid onto the filter paper, letting it soak briefly, removing the air bubbles, then pouring off the excess. Ensure that there is no pooled liquid on the filter paper prior to placing membrane.

*It is important to use filter paper prewetted but not saturated with the appropriate solutions, to prevent colonies from over-swelling and losing their circular shape.*

8. Preheat 10% SDS, 3× SSC, and the Pyrex dishes with the prewetted filter paper to 65°C.

9. Place membrane colony-side-up on the SDS-prewetted filter paper in the corresponding dish, cover the membrane with Saran Wrap or equivalent plastic wrap to prevent filter paper from drying out, and incubate 5 min at 65°C.

10. Transfer the membrane colony-side-up to the filter paper prewetted with 3× SSC, cover membrane with Saran wrap or equivalent plastic wrap to prevent the filter paper from drying out, and incubate 5 min at 65°C.

11. Transfer membranes to dry filter paper and allow to air dry 10 min at room temperature.

12. Bake membranes 15 min in a 70°C oven.

**Perform RNA colony-blot hybridization**

13. Wash membranes three times in 150 ml prewashing solution for 15 min at 37°C.

14. Wash membranes in 150 ml prewashing solution at 60°C, with agitation in a shaking water bath.

15. Rinse membranes in DEPC-treated water.

16. Prehybridize the membranes 30 min at 60°C in RNA colony blot hybridization solution at a ratio of 10 ml solution per 100-cm² of blot membrane in a hybridization oven.

*85 mm-membranes have a surface area of ~60 cm².*

*RNA colony blot hybridization solution will form a precipitate when kept at room temperature. If this happens, heat to 40° to 50°C to resuspend.*

17. Prepare a sufficient quantity of probe-containing RNA colony blot hybridization solution by adding 32 µl of 25 ng/µl fluorescein-labeled Vchomim1276 probe per 10 ml of RNA colony blot hybridization solution. At the end of the incubation in step 16, pour off the solution from the membrane and add the probe-containing RNA colony blot hybridization solution at a ratio of 10 ml solution per 100-cm² blot.

18. Hybridize overnight (ideally for 16 to 20 hr) at 60°C.

19. After hybridization, wash membranes 30 min at 60°C in washing solution for colony blot hybridization with fluorescein-labeled probes.

20. View/image the membrane using Typhoon Scanner or Dark Reader.

21. From replica plates, subculture colonies that were positive by blot hybridization for further analysis, if desired.
Colony Blot Hybridization Using DIG-Labeled ctxA DNA Probe

The previous colony blot hybridization protocol (Basic Protocol 6) is used to detect *V. cholerae* and closely related *V. mimicus*. This protocol, on the other hand, targets only toxigenic strains of *V. cholerae*. The presence of ctxA is confirmed by hybridization using a ctxA-specific DNA probe. There may be some cross-reactivity of the probe with the heat-labile toxin (LT) of *E. coli* (Dallas and Falkow, 1980). Colony blots are prepared according to the method of Pal et al. (1992). The hybridization is done according to the DIG protocol. Readers are encouraged to consult the manual accompanying the High Prime kit (Roche); probe-labeling is described in the Support Protocol. The ctxA probe can be produced by PCR, using the pCTA primer set (see Table 6A.5.4), or by *Eco*RI digestion of plasmid, pKT-901, which contains a 540-bp *Xba*I-Clal fragment of ctxA (Kaper et al., 1988).

**Additional Materials (also see Basic Protocol 6)**

- Lysis buffer: 0.5 M NaOH/1.5 M NaCl (prepare fresh)
- Neutralization solution 0.5 M Tris-Cl, pH 7.2 (APPENDIX 2A)/1.5 M NaCl (store up to 6 months at 2° to 8°C)
- 1 × SSC (APPENDIX 2A)
- 40 µg/ml proteinase K in 1 × SSC (store up to 2 months at –20°C)
- DIG High Prime DNA Labeling and Detection Starter Kit II: (Roche) including:
  - DIG Easy Hybridization Buffer
  - 10× blocking solution
  - Antibody: anti-digoxigenin–AP conjugate
  - CSPD
- DIG-labeled ctxA probe (Support Protocol)
- Stringency wash solution I: 2 × SSC (APPENDIX 2A)/0.1% (w/v) SDS
- Stringency wash solution II: 0.5 × SSC (APPENDIX 2A)/0.1% (w/v) SDS
- Washing solution for colony blot hybridization with DIG-labeled probes (see recipe)
- Maleic acid buffer (see recipe)
- Detection buffer: 0.1 M Tris-Cl, pH 9.5 (APPENDIX 2A)/0.1 M NaCl
- Sterile nylon (or nitrocellulose) membranes, 85 mm, 0.22-µm (GE Water and Process Technologies)
- UV cross-linker or transilluminator
- 42° and 65°C shaking incubators
- Room temperature shaking water bath
- Boiling water bath
- Hybridization pouches
- X-ray film (Kodak or Fuji) and developing facility

**Prepare blots**

1. Prepare LB agar spread plates as described in Basic Protocol 6, step 1.

2. Incubate plates overnight at 37°C

3. Using a pencil, mark each membrane on the top side with the blot ID (e.g., recording the medium, sample, and dilution) that matches the plate to be lifted. Also place asymmetrical orientation marks on the membrane to facilitate later comparison between membrane and plate.

4. Overlay the corresponding membrane onto each of the incubated spread plates, starting from the center to ensure there are no air bubbles.

5. Allow at least 15 min for transfer.
6. Transfer the blot onto a new LB plate keeping colony side up and incubate 3 hr at 37°C. Wrap the master plate with Parafilm and keep at 10° to 15°C.

   Master plates can be maintained in this manner for up to 2 weeks.

7. Place membranes, colony-side-up, onto Whatman no. 3 filter paper prewetted with lysis buffer in a Pyrex dish (see Basic Protocol 6, step 7, for additional considerations). Incubate 10 min at room temperature.

   It is important to use filter paper prewetted but not saturated with the appropriate solutions, to prevent colonies from over-swelling and losing their circular shape.

8. Remove the membrane from lysis buffer and place onto Whatman no. 3 filter paper prewetted with neutralization solution in a Pyrex dish. Incubate 5 min at room temperature.

9. Transfer membrane to a fresh filter paper prewetted with neutralization solution and incubate 5 min at room temperature.

10. Remove membrane from neutralization solution, place onto dry no. 3 Whatman filter paper and allow to air dry (~15 min) at room temperature, but not to complete dryness.

11. Immobilize colonies onto the membrane using a UV cross-linker or transilluminator.

   Damp membranes should be cross-linked at an output intensity of 120-mJ/cm², which corresponds to the optimal or auto-cross-link setting on commercially available machines. Transilluminators or hand-held UV lamps can be used, if calibrated; however, the following times should be sufficient: 1 min for 254-nm lamps or 3 min for 302-nm lamps.

12. Rinse the blot twice in 1× SSC buffer and air dry (~30 min).

13. Treat membranes 30 min at 42°C with 100 ml of 40 μg/ml proteinase K solution using gentle shaking.

14. Rinse filters three times in 1× SSC in a shaking water bath at room temperature, each time for 10 min. Allow to air dry (~30 min) at room temperature.

Prehybridize and hybridize

15. Preheat DIG-Easy Hybridization buffer to 42°C.

16. Prehybridize blots in preheated DIG-Easy Hybridization buffer for 30 min at 42°C with gentle agitation.

17. Denature the DIG-labeled ctxA probe by boiling for 5 min and rapidly cooling on ice. Prepare 25 ng/ml denatured probe in DIG Easy Hybridization solution.

18. Pour off prehybridization solution.

19. Add freshly prepared DIG Easy Hybridization Solution plus 25 ng/ml denatured probe and incubate overnight at 42°C.

Perform stringency washes

20. Pour off probe-containing hybridization solution.

   Probe-containing hybridization solution can be reused several times. Store up to 2 months at –20°C.

21. Wash membrane twice in stringency wash solution I for 5 min at room temperature under constant agitation.

22. Wash membrane twice in stringency wash solution II for 15 min at 65°C under constant agitation.
**Detect positive colonies**

23. Rinse membrane 5 min in washing solution for colony blot hybridization with DIG-labeled probes.

24. Dilute the 10× blocking solution from the kit to 1× in maleic acid buffer. Incubate the membrane 30 min at 25°C in 1× blocking solution (from kit; prepare according to manufacturer’s instructions).

25. Prepare antibody solution by diluting the anti-digoxigenin–AP conjugate from the kit 1:10,000 in 1× blocking solution (prepared as in the previous step). Incubate membrane 30 min at 25°C in this diluted antibody solution for 30 min.

26. Wash the membrane twice, each time for 15 min, in solution for colony blot hybridization with DIG-labeled probes, for 15 min each.

27. Incubate membrane in detection buffer for 5 min at room temperature (25°C).

28. Place membrane in hybridization pouch. Add CSPD (from kit) to the membrane, cover and incubate at room temperature for 5 min.

29. Remove excess liquid from pouch, seal, and incubate at 37°C for 10 min.

30. Expose the membrane to X-ray film for 20 min at room temperature and develop.

*If film is under- or overexposed, repeat, varying time of exposure accordingly.*

31. From master plates (step 6), subculture colonies that were positive by blot hybridization for further analysis.

**SUPPORT PROTOCOL**

Digoxigenin Labeling of ctxA Probe Using DIG High Prime Kit

PCR- or restriction digestion–generated probes can be labeled using the DIG High Prime kit (Roche), which is based on randomly incorporating digoxigenin-11-dUTP. A 563-bp ctxA fragment can be produced by amplifying extracted DNA from a toxigenic laboratory reference strain. The PCR product should be purified by using a PCR clean-up kit or by gel electrophoresis and extraction. Alternatively, a PCR product may be labeled during the PCR amplification using the PCR DIG Probe Synthesis Kit (Roche). A 554-bp ctxA probe is also available on a plasmid, pKTN901 (Kaper et al., 1988), which has been widely used (Pal et al., 1992; Islam et al., 2005). The XbaI-ClaI fragment can be removed from the plasmid by digestion with EcoRI, and gel purified. Labeled probes can then be used in the DIG-based hybridization as described in Alternate Protocol 2.

**Materials**

- DNA extracted from toxigenic (ctxA+) *V. cholerae* reference strain (ATCC)
- Forward and reverse pCTA primers for amplifying ctxA (Table 6A.5.4)
- Plasmid pKTN901 (available from Dr. James Kaper, jkaper@umaryland.edu) or other source of ctxA probe
- *Eco*RI restriction endonuclease (or other restriction enzyme depending on source of ctxA probe) and restriction buffer
- 5× DIG High Prime labeling mixture (Roche)
- 0.2 M tetrasodium EDTA, pH 8.0
- Boiling water bath
- 65°C water bath
- Additional reagents and equipment for purifying DNA fragments (Moore et al., 2002)
1. To generate ctxA target fragment by PCR: Amplify extracted DNA of a toxigenic (ctxA⁺) V. cholerae reference strain using the pCTA primer set.

2. To generate ctxA target fragment by restriction digestion: Digest plasmid pKTN901 with EcoRI for 2 hr at 37°C or digest other source of ctxA probe using restriction enzyme and conditions appropriate to construct.

3. Clean up PCR reaction product or digestion product by gel electrophoresis followed by gel extraction (Moore et al., 2002).

4. In a microcentrifuge tube, bring 1 µg purified ctxA fragment up to a total volume of 16 µl with sterile distilled water.

5. Denature by placing in a boiling water bath for 10 min, then rapidly transfer to an ice/water bath and chill 5 min.

6. Add 4 µl of 5× DIG High Prime labeling mixture to the DNA solution, mix, then microcentrifuge briefly to collect liquid at the bottom of the tube.

7. Incubate overnight at 37°C.

8. Stop reaction by adding 2 µl of 0.2 M tetrasodium EDTA and/or by heating to 65°C for 10 min.

IMMUNOLOGICAL METHODS FOR DETECTION OF V. CHOLERAE

Conventional culture methods are ineffective when bacterial cells have entered into the viable but nonculturable (VBNC) state. Thus, direct or indirect detection of the cells themselves becomes extremely important. The discovery of monoclonal antibodies in the 1980s and, subsequently, the development of a monoclonal antibody against V. cholerae O1,s triggered development of direct detection methods for this bacterial species (Xu et al., 1984; Hasan et al., 1992). Using immunological methods, the mystery concerning the inability to culture V. cholerae in environmental samples during inter-epidemic periods in Bangladesh was resolved by the discovery of VBNC V. cholerae (Roszak and Colwell, 1987; Huq et al., 1990).

Direct Fluorescent Antibody–Direct Viable Count (DFA-DVC) Method

The direct fluorescent antibody staining method for rapid detection of V. cholerae serogroup O1 and O139 is a very useful, direct, and culture-independent method. Coupled with the direct viable count method of Kogure et al. (1979), it can distinguish culturable, viable cells from viable but nonculturable cells (VBNC) of V. cholerae (Chowdhury et al., 1995). It is a two-step method in which samples are incubated with yeast extract in the presence of nalidixic acid, after which actively viable, substrate-responsive cells become enlarged and elongated (Kogure et al., 1979). Next, smears prepared from this suspension on a glass slide are stained with fluorescently labeled monoclonal antibody raised against the “A” factor of V. cholerae O1 lipopolysaccharide, which reacts with both serotype Ogawa and serotype Inaba (Colwell et al., 1990; Hasan et al., 1994). Antibodies against V. cholerae O139 are also available (Hasan et al., 1995). When observed under an epifluorescent microscope, elongated cells of V. cholerae O1 or O139 (based on the type of antibody used) exhibit a bright green–fluorescing periphery (the outer cell wall) with a dark interior (Fig. 6A.5.4). V. cholerae O1- and O139 DVC-DFA-positive samples can be confirmed by PCR (Binsztein et al., 2004). DFA-DVC is a rapid method by which one can determine the presence of V. cholerae within 8 hr (when the DVC incubation is 6 hr); however, overnight incubation with yeast extract and nalidixic acid is preferred. Kits for V. cholerae O1 (Cholera DFA) and O139 (Bengal DFA) DFA tests are commercially available (New Horizon Diagnostics).
Detection, Isolation, and Identification of V. cholerae from the Environment

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Figure 6A.5.4  DFA staining of V. cholerae O1 using the Cholera DFA Kit (New Horizon Diagnostics). (A) Fresh culture; (B) VBNC cells; (C) and (D), DVC-incubated cells. For the color version of this figure go to http://www.currentprotocols.com.

Materials

Concentrated water or homogenized plankton sample (Basic Protocol 2)
2.5% (w/v) yeast extract in distilled water
0.2% (w/v) nalidixic acid in distilled water
37% to 40% formaldehyde solution or fresh 4% formaldehyde prepared from paraformaldehyde
Absolute methanol
Cholera DFA and/or Bengal DFA kit (New Horizon Diagnostics) containing:
   FITC-conjugated DFA reagent
   Positive and negative control
   Fluorescent mounting medium
   Phosphate-buffered saline (PBS; APPENDIX 2A)
   Humidified chamber; 50-ml conical centrifuge tube containing 1 to 2 strips of water-saturated filter paper
   Multiwell slides and coverslips
   Epifluorescent microscope with FITC filter set

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All solutions should be filter-sterilized through a 0.1-µm filter, as VBNC cells of V. cholerae appear as small coccoid cells in a size range of 0.1 to 0.8 µm.

Perform DVC incubation

1. To 1 ml concentrated water or homogenized plankton sample (Basic Protocol 2), add 10 µl of 2.5% yeast extract and 10 µl of 0.2% nalidixic acid.

   The water sample can be concentrated by filtration through a 0.22-µm filter followed by resuspension or by centrifugation for 10 min at 12,000 × g, room temperature, followed by removal of the supernatant and resuspension.

   Freeze a parallel 1-ml sample for PCR confirmation (see Basic Protocol 5).
2. Incubate the mixture at room temperature (25°C) for a minimum of 6 hr to overnight.

3. Fix the sample by adding formaldehyde to a final concentration of 3% (v/v) and incubating 30 min at room temperature in the dark.

   *Fixed samples can be stored at 4°C in the dark for up to 6 months.*

**Perform DFA protocol**

4. Place 5 to 10 µl of the fixed sample onto a glass slide and air dry ~15 to 20 min.

5. Fix by adding 5 µl absolute methanol and air dry 1 to 5 min.

6. Add 10-µl of reconstituted FITC-conjugated specific DFA reagent from the Cholera DFA or Bengal DFA kit.

   *Use of this kit is discussed in Hasan et al. (1994).*

7. Incubate 30 min at 37°C in a humidified chamber. Protect slide from light.

8. Rinse slide with ~50 ml PBS. Air dry slide in the dark ~15 to 20 min.

9. Mount slide with one drop of fluorescent mounting medium and add coverslip.

10. Observe under an epifluorescent microscope (see Fig 6A.5.4).

**Indirect Fluorescent Antibody (IFA) Method**

This immunofluorescent method for detection of *V. cholerae* serogroup O1 in aquatic environmental samples was first introduced by Xu et al. (1984). Antiserum specific for O1 somatic antigen produced in rabbits was used with fluorescein isothiocyanate (FITC)–conjugated goat anti–rabbit immunoglobulin serum, with rhodamine isothiocyanate (RTIC)-conjugated bovine serum albumin as the background stain. This indirect staining method was found to be very useful for detecting organisms in samples that gave negative results by culture and was subsequently modified to a direct method (Brayton et al., 1986; Huq et al., 1990). The direct fluorescent antibody method was later optimized (Hasan et al., 1994) and packaged as the Cholera DFA Kit (New Horizon Diagnostics, Inc.). The IFA protocol remains useful for laboratories where commercial DFA kits for *V. cholerae* are not readily available.

**Materials**

- Concentrated water or homogenized plankton sample (Basic Protocol 2)
- 95% ethanol
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- FA Rhodamine counterstain (Becton Dickinson)
- Polyvalent *V. cholerae* O1-specific antiserum (BD Bioscience)
- FITC-conjugated anti-rabbit globulin goat serum (Sigma)
- Low-fluorescence antiquenching mounting medium: e.g., FA (BD Biosciences) or Citifluor AF1/AF3 (Electron Microscopy Sciences)
- Multiwell, Teflon-coated slides
- 35° and 55°C incubators
- Humidified chamber: 50-ml conical centrifuge tube containing 1 to 2 strips of water-saturated filter paper
- Glass coverslips
- Epifluorescent microscope with FITC bandwidth filter

**Prepare and fix samples**

1. Add an appropriate amount (dependent on concentration of sample and well size) of each sample to be tested to a Teflon-coated multiwell slide. Air dry 15 to 20 min at room temperature.
2. Fix the sample by adding 95% ethanol to each sample-containing well. Air dry 5 to 10 min at room temperature.

3. Heat slide 10 min in a 55°C incubator.

   *Slides may be stored up to 1 month at −70°C at this point.*

**Stain samples**

4. Rinse the slide(s) with ~50 ml PBS and air dry 15 to 20 min.

5. While slide is drying, equilibrate humidified chamber in 35°C incubator ~15 min.

6. To each dry sample well, add 1 to 2 drops of a 1:20 dilution of FA Rhodamine in distilled water. Incubate in humidified chamber 30 min at 35°C.

   *Minimize exposure to light from this step forward.*

7. Rinse slide by gently flooding with ~50-ml PBS, then soak in PBS 10 min at room temperature. Remove slide from PBS and rinse again briefly in PBS.

8. Allow slide to air dry 15 to 20 min.

9. Add 5 to 10 µl of *V. cholerae* O1-specific antiserum. Incubate in humid chamber 30 min at 35°C.

10. Repeat steps 7 and 8 using fresh PBS for washing.

11. Add 1 to 2 drops undiluted FITC-conjugated anti-rabbit globulin goat serum and incubate in humidified chamber 30 min at 35°C.

12. Repeat washing steps 7 and 8 using fresh PBS.

**Mount and examine slides**

13. Mount each slide with a glass coverslip and a low fluorescence, anti-quenching mounting medium, such as Citifluor AF1.

14. Examine samples immediately using a epifluorescent microscope with a FITC band-pass filter.

   *Slides can be stored in the dark up to 1 to 2 days at 4°C.*

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Alkaline peptone water (APW)**

10 g peptone
10 g sodium chloride
Add 800 ml H$_2$O
Adjust pH to 8.6 with NaOH
Adjust volume to 1 liter with water
Autoclave 15 min at 121°C
Store up to 6 months at 2° to 8°C

**CTAB/NaCl Solution**

Add 4 g NaCl to 80 ml water and dissolve. Slowly add 10 g cetyltrimethylammonium bromide (CTAB) while heating and stirring at 65°C. Adjust volume to 100 ml with water. Store up to 6 months at room temperature.
**Gelatin agar**

4 g neopeptone  
1 g yeast extract  
5 g sodium chloride  
15 g gelatin  
15 g agar  
Adjust volume to 1 liter with water  
Autoclave to sterilize  
Store up to 1 month at 2° to 8°C

**Kligler iron agar**

Combine the following:  
20 g polypeptone  
10 g lactose  
1 g glucose  
5 g sodium chloride  
0.5 g ferric ammonium citrate  
0.5 g sodium thiosulfate  
0.025 g phenol red  
15 g agar  
Adjust volume to 1 liter with water  
Boil to dissolve agar completely  
Dispense 5-ml portions of the medium into 16 × 125–mm tubes (other size tubes can be used, adjust dispensed media volume to about 1/3 volume of test tube). Autoclave 15 min at 121°C. Allow tubes to solidify in a slanted position to give a generous butt (underneath a 20° to 40° slant). Store up to 6 months at 2° to 8°C.  
*This medium is also available commercially as a dehydrated powder from Difco (BD Biosciences).*

**Maleic acid buffer**

0.1 M maleic acid  
0.15 M sodium chloride  
Adjust pH to 7.5 with solid NaOH  
Prepare fresh

**Methyl Red/Voges-Proskauer (MR-VP) medium**

7 g peptone  
5 g glucose  
5 g potassium phosphate, dibasic  
Adjust pH to 6.9 with HCl  
Adjust volume to 1 liter with water  
Autoclave to sterilize  
Store up to 6 months at 2° to 8°C  
*This medium is also available commercially as a dehydrated powder from Difco (BD Biosciences).*

**Modified nutrient agar**

3 g beef extract  
5 g peptone  
10 g sodium chloride  
15 g agar  
*continued*
Adjust volume to 1 liter with water
Autoclave
Allow medium to cool to 55°C, then pour plates
Store up to 1 month at 2° to 8°C

**Moeller decarboxylase broth base**

- 5 g peptone
- 5 g beef extract
- 0.5 g dextrose
- 0.01 g bromocresol purple
- 0.005 g Cresol red
- 0.005 g pyridoxal

Adjust volume to 1 liter with water
Boil 1 min to dissolve
Add 10 g L-amino acid or 20 g DL-amino acid as specified in protocol
If adding ornithine, adjust pH to 6.0 with HCl
Autoclave to sterilize
Store up to 6 months at 2° to 8°C

*This medium is also available commercially as a dehydrated powder from Difco (BD Bioscience).*

**Oxidase reagent**

Dissolve 0.05 g \( N,N,N',N' \)-Tetramethyl-p-phenylenediamine dihydrochloride into 5 ml water. Prepare fresh

*Final concentration is 1% (w/v).*

**Prewashing solution**

Prepare the following in DEPC-treated water (**APPENDIX 2A**)

- 3× SSC (**APPENDIX 2A**), RNase-free
- 0.1% (w/v) SDS RNase-free

Prepare fresh

**Purple broth base**

- 10 g proteose peptone no. 3
- 1 g beef extract
- 5 g sodium chloride
- 0.015 g bromocresol purple

Dissolve in 1 liter cold water
Add carbohydrate (as specified in protocol) to 1% (w/v) final concentration
Autoclave to sterilize
Store up to 6 months at 2° to 8°C

*This medium is also available commercially as a dehydrated powder from Difco or BBL (both trademarks of BD Bioscience).*

**RNA colony blot hybridization solution**

Prepare the following in DEPC-treated water (**APPENDIX 2A**)

- 0.9 M NaCl
- 50 mM sodium phosphate, pH 8.0 (**APPENDIX 2A**)
- 5 mM tetrasodium EDTA
- 0.5% (w/v) SDS

Store up to 1 month at room temperature

Precipitation will occur upon standing at room temperature. Heat to 45° to 50°C to resuspend.
Tellurite taurocholate gelatin agar (TTGA) plates

10 g tryptone
10 g sodium chloride
5 g sodium taurocholate
1 g sodium carbonate
30 g gelatin
15 g agar
Adjust volume to 1 liter with water
Boil to completely dissolve ingredients
Final pH should be 8.5; if not, adjust with HCl
Autoclave and add potassium tellurite to 1% (w/v) final concentration
Allow medium to cool to 55°C, then pour plates
Store up to 1 month at 2° to 8°C

Thiosulfate citrate bile-salts sucrose (TCBS) agar

5 g yeast extract
10 g peptone
10 g sodium thiosulfate
10 g sodium citrate
8 g ox bile
20 g sucrose
10 g sodium chloride
1 g ferric citrate
0.04 g bromthymol blue
0.04 g thymol blue
14 g agar
Adjust volume to 1 liter with H2O
Boil to completely dissolve; do not autoclave
Allow medium to cool to 55°C, then pour plates
This medium is also available commercially as a dehydrated powder from Oxoid.

IMPORTANT NOTE: TCBS medium is never autoclaved. Sterility is achieved (for practical purposes) by the selective ingredients added to the medium. The high concentrations of thiosulfate and citrate and the strong alkalinity of this medium largely inhibit the growth of Enterobacteriaceae. Ox bile and cholate suppress primarily enterococci. Any coliform bacteria, which may grow, cannot metabolize sucrose. Only a few sucrose-positive Proteus strains can grow to form yellow, Vibrio-like colonies. The mixed indicator thymol blue/bromthymol blue changes its color to yellow, when acid is formed, even in this strongly alkaline medium.

Washing solution for colony blot hybridization with DIG-labeled probes

0.1 M maleic acid
0.15 M sodium chloride
0.3% (v/v) Tween 20
Adjust pH to 7.5 with solid NaOH
Prepare fresh

Washing solution for colony blot hybridization with fluorescein-labeled probes

Prepare the following in DEPC-treated water (APPENDIX 2A)
1× SSC (APPENDIX 2A)
0.1% SDS
Prepare fresh
**COMMENTARY**

**Background Information**

Although microorganisms were isolated and characterized a century ago, the past three decades could be termed as the “golden age of environmental microbiology” (Leadbetter, 1997). Twenty years ago, some microbiologists estimated that perhaps 40% of all prokaryotes were recognized and understood. However, today, it would be overly optimistic to suggest a number even as high as 5%, because new methods employed in environmental microbiology have shown that many microorganisms—perhaps <1% of the microbial species in the world oceans (Venter et al., 2004)—have been cultured. Molecular probes are considered the ultimate tools for detection and characterization of microorganisms (Woese, 1994; Amann et al., 1995) without the need for culture, but probes are designed only on the basis of existing knowledge of molecular signatures of organisms that were obtained originally in pure culture (Leadbetter, 1997). The study of environmental samples is very different from clinical microbiology. The diversity of microorganisms inhabiting highly dissimilar environments is enormous. Therefore, no unique test or set of tests will be appropriate for every environment. Detecting a particular organism will depend on environmental conditions during sample collection. Investigators would be wise first to determine the kind of data or results that will be needed and the appropriateness of available methods, as well as the feasibility of carrying out the procedures to achieve the goal. If a target pathogen is not detected in a sample using a particular method or even by a set of methods, it is risky to conclude that the pathogen is not present, especially if there are other indications that it may be present. Like any bacteriological test, replication and accurate interpretation are critical to achieve reproducible results. Most importantly, it is the responsibility of the investigator to choose appropriate tests and carefully design the study, including controls; otherwise, data interpretation will be very difficult, with misleading conclusions.

*Vibrio cholerae* is an autochthonous member of estuarine and other aquatic communities (Colwell et al., 1977; Kaper et al., 1979; Colwell et al., 1981; Colwell and Spira, 1992) and the disease with which it is associated, cholera, is largely a waterborne disease. *V. cholerae* also frequently isolated from higher aquatic organisms, such as plankton (Huq et al., 1990; Tamplin et al., 1990; Islam et al., 1993), oysters (Hood et al., 1981; Murphree and Tamplin, 1991), and water hyacinth (Spira et al., 1981). Transmission of cholera occurs predominantly by the fecal-oral route, via ingestion of contaminated water or contaminated seafood. Endemic areas include Southeast Asia, Africa, and Latin and South America, where poverty and poor sanitation are common.

Of the over 200 recognized serogroups of *V. cholerae* (Yamai et al., 1997), only the O1 and O139 serogroups cause epidemic and pandemic cholera outbreaks. Predominantly, non-O1/non-O139 *V. cholerae* strains are isolated from the environment, even in epidemic areas. Of the O1 serogroup strains that are isolated from the environment, most are nonpathogenic; i.e., missing the genes necessary for pathogenicity (ctxA−, tcpA−; Roberts et al., 1982; Minami et al., 1991).

*V. cholerae*, along with an ever-increasing number of species of Gram-negative bacteria, is capable of entering a state of dormancy, commonly termed viable but nonculturable (VBNC or sometimes VNC; Roszak and Colwell, 1987; Colwell, 1991; Colwell and Huq, 1994). It is becoming apparent that this state is an important survival strategy and perhaps an essential part of the life cycle of these bacteria. Microcosm experiments using VBNC *V. cholerae* suggest that a large number of environmental isolates exist in this state as compared to culturable cells (Huq et al., 1990; Huq et al., 2000).

**Critical Parameters and Troubleshooting**

Successful detection and/or isolation of *V. cholerae* may depend upon many environmental variables in addition to the appropriateness of the method used. For example, chances of detection of *V. cholerae* O1 are higher in warmer months when the water temperature is >30°C (Louis et al., 2003; Binsztein et al., 2004). The conventional culture method presented in this unit (Basic Protocol 2) is the most common, and preferred, culture-based protocol used to isolate *V. cholerae* (Morris et al., 1979; Rennels et al., 1980). There have been a number of proposed derivatives of this technique (Basic Protocol 2), based largely on alternative media. For enrichment, alkaline bile peptone water (Spira et al., 1981), Monsur’s tellurite taurocholate broth (Monsur, 1961), and sodium-gelatin phosphate broth (Rennells et al., 1980) have been proposed. A second
enrichment step may be used, but this will add an additional 6 to 8 hr to an already lengthy protocol. Equally, a number of differential or selective plating media have been proposed (Tamura et al., 1971; Chatterjee et al., 1977; Morris et al., 1979; Shimada et al., 1990). Spira and Ahmed (1981) reported that using Moore swabs may increase the number of V. cholerae isolated over concentration by membrane filtration; however, membrane filtration remains the favored method. The authors of this unit include a subculturing step on to nonselective medium—modified nutrient agar or gelatin agar. This step serves two purposes. First, it is critical that pure colonies be achieved before proceeding with a battery of biochemical tests; mixed cultures will give ambiguous results. Second, growth on TCBS is not satisfactory for the oxidase test or for serotyping. When characterizing by biochemical tests, the authors present eleven biochemical tests to be performed, in addition to the oxidase test (Table 6A.5.2). This list is not definitive; the battery can be increased or decreased, and/or modified.

It is well documented that PCR amplification of environmental samples can be inhibited by dissolved organics, such as humic acid. For that reason, it is suggested the DNA be extracted first, which adds only 3 to 4 hr to the protocol. For all direct PCR examinations, include the eubacterial PCR reaction to confirm template quality on samples that are negative for V. cholerae gene targets.

Colony blot hybridization (Basic Protocol 6 and Alternate Protocol 2) is presented here as a culture-based alternative to the conventional culture method described in Basic Protocol 2. At least three serial-fold dilutions should be spread-plated to ensure screening of an ideal number of colonies (50 to 200). As an alternative, the authors suggest filtering water directly through the membrane (0.22-µm) to be used for the colony lift, although some knowledge of the microbial density of the water sample is needed for this procedure. For the V. cholerae-specific RNA-colony blot/hybridization, care should be taken to eliminate RNase contamination. Diethylpyrocarbonate (DEPC; APPENDIX 2A) is used to eliminate the otherwise stable, ubiquitous enzymes. The ctxA-DNA colony blot hybridization (Alternate Protocol 2) is presented for cases in which only toxigenic V. cholerae is investigated. This method has two drawbacks: cross-reaction with heat labile enterotoxin (LT) of E. coli may occur, and it is relatively expensive if a large number of samples are to be analyzed.

DVC-DFA (Basic Protocol 7) and IFA (Basic Protocol 8) of V. cholerae are relatively rapid protocols compared to the other methods presented. One pitfall of these immunofluorescence methods is the confounding observation of autofluorescing constituents found in environmental water samples. All “positive” samples should be documented by digital or film photography; confirmation by PCR of a parallel sample (frozen, but not fixed) is advisable.

Table 6A.5.5 outlines some of the more common problems that may be experienced in performing the basic and alternate protocols from this unit. This is not an exhaustive list; others may be encountered. Consult the troubleshooting sections from the referenced units of Current Protocols in Molecular Biology (see Literature Cited) for further advice.

**Anticipated Results**

V. cholerae can be easily isolated from estuarine environments during the warm summer months—even in nonepidemic areas. The chance of successful isolation decreases during the colder winter months, even from samples that are positive by PCR or DFA. Figure 6A.5.2 shows the typical growth of V. cholerae on TCBS and TTGA media. Growth on TCBS by V. cholerae appears as small (1- to 3-mm diameter) flat, yellow colonies (Fig. 6A.5.2A). V. cholerae appear as medium to large (2- to 5-mm in diameter) flat, translucent colonies on TTGA (Fig. 6A.5.2B). There will be a zone of clearing (or halo) surrounding V. cholerae colonies due to hydrolysis of gelatin. A dark center usually develops after 24 hr. All strains of V. cholerae can exhibit various colony morphologies, including a rugose form (White, 1940). The strain in Figure 6A.5.2A exhibits two colony morphologies, translucent and opaque. For the sake of comparison, several different positive-control V. cholerae strains should be subcultured at the time of subculturing from APW to selective medium. The selective media TCBS and TTGA still allow the growth of other Vibrio species and related bacteria. For example, V. parahaemolyticus will appear as blue-green colonies, V. mimicus will appear as green colonies, and V. alginolyticus as larger, yellow colonies on TCBS. Therefore, colonies growing on these media are not necessarily all V. cholerae and are labeled “presumptive.” The goal of each step in the biochemical identification method is to reduce the number of suspected or presumptive colonies, which will include other Vibrio species and related Vibrionaceae, until
<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
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<tbody>
<tr>
<td><strong>Basic Protocol 1</strong></td>
<td></td>
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<tr>
<td>Little or no plankton in cod-end collecting bucket</td>
<td>Check pore size of plankton net and ensure that it is 64 µm</td>
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<td></td>
<td>Zooplankton should be sampled near dawn or dusk (1-2 hr after sunrise or before sunset) when they are nearer to the surface</td>
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<td>Filter more water through plankton net</td>
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<tr>
<td><strong>Basic Protocol 2</strong></td>
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<tr>
<td>Failure of positive control to give correct results for biochemical tests</td>
<td>Use multiple positive controls</td>
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<td></td>
<td>Prepare multiple replicates for each positive control</td>
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<tr>
<td>Results of biochemical tests difficult to interpret</td>
<td>Ensure proper incubation temperature and time</td>
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<td>Tests giving variable or weakly positive result should be replicated</td>
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<td></td>
<td>For decarboxylase tests, ensure that a tube without amino acid is used, to demonstrate growth with negative result</td>
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<td></td>
<td>For esculin hydrolysis, use presence/loss of fluorescence as measure of negative/positive</td>
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<tr>
<td><strong>Basic Protocol 3</strong></td>
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<tr>
<td>Autoagglutination or clumping in saline without antisera</td>
<td>“Rough” morphotypes cannot be serogrouped with antisera; use O1/O139 rfb PCR primers with Basic Protocol 4 to test for toxigenic serogroups</td>
</tr>
<tr>
<td><strong>Basic Protocol 4</strong></td>
<td></td>
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<tr>
<td>No PCR product with positive control</td>
<td>Ensure all components are added to reaction at the proper concentration</td>
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<td></td>
<td>Use fresh dNTPs</td>
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<td></td>
<td>Prepare fresh crude template of positive control as it will degrade over time</td>
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<td></td>
<td>Dilute crude template 1:5000 or more and repeat the control reaction</td>
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<td></td>
<td>Quantify crude template by gel electrophoresis (~10 µl) to ensure sufficient template concentration</td>
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<tr>
<td>PCR product with negative control</td>
<td>Most likely caused by carryover contamination in one of the reaction components; make new components</td>
</tr>
<tr>
<td><strong>Basic Protocol 5</strong></td>
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<tr>
<td>No PCR product with positive control</td>
<td>Ensure all components are added to reaction at the proper concentration</td>
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<tr>
<td></td>
<td>Use fresh dNTPs</td>
</tr>
<tr>
<td>PCR product with negative control</td>
<td>Most likely caused by carryover contamination in one of the reaction components; make new components</td>
</tr>
<tr>
<td><strong>Alternate Protocol 1</strong></td>
<td></td>
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<tr>
<td>No PCR product with positive control</td>
<td>Ensure all components are added to reaction at the proper concentration</td>
</tr>
<tr>
<td></td>
<td>Use fresh dNTPs</td>
</tr>
<tr>
<td>PCR product with negative control</td>
<td>Most likely caused by carryover contamination in one of the reaction components; make new components</td>
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*Continued*
Table 6A.5.5  Troubleshooting Guide for V. cholerae Isolation and Detection, Contd

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
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<tbody>
<tr>
<td><strong>Basic Protocol 6</strong></td>
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<tr>
<td>Positive control is negative</td>
<td>Ensure solutions used are RNase-free</td>
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<td></td>
<td>Increase incubation time of lysis step, especially if colonies are &gt;3 mm</td>
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<td>Ensure that the correct microscope filters are used for fluorochrome selected (other fluorochromes may be used)</td>
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<tr>
<td>Positive control gives weak signal</td>
<td>Check scanning settings on detection instrument</td>
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<td></td>
<td>Increase probe concentration and/or hybridization time</td>
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<tr>
<td><strong>Alternate Protocol 2</strong></td>
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<tr>
<td>Positive control is negative or weak</td>
<td>Ensure that solutions are used in correct order</td>
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<tr>
<td></td>
<td>Overexposure to UV source will degrade DNA template; consider using positively charged nylon membranes, which do not need cross-linking</td>
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<tr>
<td></td>
<td>Extend hybridization time</td>
</tr>
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<td></td>
<td>Extend development time</td>
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<tr>
<td>Positive control is overdeveloped or background is high</td>
<td>Check hybridization temperature</td>
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<td></td>
<td>Do not allow membrane to dry</td>
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<tr>
<td></td>
<td>Decrease development time</td>
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<tr>
<td><strong>Support Protocol</strong></td>
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<tr>
<td>Positive control from Alternate Protocol 2 is negative or weak</td>
<td>Check efficiency of probe labeling reaction</td>
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<td></td>
<td>Increase probe concentration (&gt;25 ng/ml)</td>
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<tr>
<td><strong>Basic Protocol 7</strong></td>
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<tr>
<td>Positive control is negative</td>
<td>Ensure that the proper filter set is used on fluorescent microscope</td>
</tr>
<tr>
<td></td>
<td>Bengal DFA (V. cholerae O139) kit positive control is sometimes poor; prepare positive control from laboratory reference strain</td>
</tr>
<tr>
<td><strong>Basic Protocol 8</strong></td>
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</tr>
<tr>
<td>Positive control signal is weak</td>
<td>Increase amount of V. cholerae O1 antiserum and FITC conjugate</td>
</tr>
</tbody>
</table>

eventually only confirmed colonies of *V. cholerae* remain. Table 6A.5.3 lists the expected results of the above prescribed biochemical tests. See Bergey et al. (1994) or Lennette et al. (1985) for more details on each of these tests. Again, it is important to include several positive controls in the biochemical test screening for comparison.

Also, the biochemical confirmation may be shortened as in the alternative “b” and “c” steps of Basic Protocol 2. Figure 6A.5.5 shows the typical reaction of *V. cholerae* on a KIA slant. Most strains of *V. cholerae* isolated from the environment will be non-O1, non-O139, and ctxA−, so serotyping usually yields few positive colonies. For O1-positive samples, the O1 serogroup can be further classified into two biotypes, namely Classical (CL) and El Tor (ET). The two biotypes are distinguished from each other on the basis of their differences in several phenotypic properties (WHO, 1987), including hemolysis (sheep red blood cells; CL−, ET+/−), hemagglutination (chicken red blood cell; CL−, ET−, ET+, Finkelstein and Mukerjee, 1963), Voges-Proskauer reaction (CL−, ET+), susceptibility to polymyxin B (CL+, ET−; Han and Khie, 1963), and sensitivity to biotype-specific phages (Nair et al., 2002). More recent studies have identified genes unique to the classical and seventh pandemic El Tor strains.

Figure 6A.5.3 shows a gel separation of the expected amplicons for several of the PCR protocols listed in the sections above. Table 6A.5.4 lists the amplicon size for all of the PCR primers mentioned in this chapter. This is not

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Detection, Isolation, and Identification of *V. cholerae* from the Environment

6A.5.34

**Figure 6A.5.5**  Growth of *V. cholerae* in a Kligler iron agar (KIA) slant. Notice the alkaline butt and acid slant, with no gas production or blackening due to hydrogen sulfide production. For the color version of this figure go to [http://www.currentprotocols.com](http://www.currentprotocols.com).

A complete listing of all PCR primers that have been designed for *V. cholerae*-specific targets. Some thought should be given to using PCR identification protocols in lieu of biochemical tests, as PCR screening will provide a definitive answer in a shorter time period.

Figure 6A.5.4 shows the typical results of *V. cholerae* with DFA (Fig. 6A.5.4A,B) and DVC-DFA (Fig. 6A.5.4C,D). DFA-stained cells of *V. cholerae* appear as having a brightly green-fluorescing periphery with a slightly dimmer interior. Fresh, vegetative cells of *V. cholerae* have a distinctive curved-rod, or vibroid shape (Fig 6A.5.4A). VBNC cells of *V. cholerae* appear as rounded, or coccoid cells, which are harder to identify than vegetative cells (Fig 6A.5.4B). The coupling of the DVC procedure (Kogure et al., 1979) with DFA staining can alleviate the difficulty in distinguishing *V. cholerae* cells from auto-fluorescing objects present in water samples.

**Time Considerations**

One important parameter to consider in screening for *V. cholerae* is how rapidly an answer is needed. For routine monitoring of environmental water sources, time is usually not as critical as in clinical cases. In clinical settings, a rapid diagnosis is critical prior to initiating antibiotic treatment and oral rehydration. Many clinical laboratories and/or hospitals employ some form of the conventional culture-based method. If the method is followed as prescribed in Basic Protocol 2, the total time before confirmation may be up to 7 days. PCR screening can be performed in a significantly shorter period of time, 6 to 24 hr, depending on the template. DFA can also be completed in a shorter period of time, 4 hr, or 24 hr if coupled with DVC. Confirmation by colony blot hybridization can be performed in 3 days. It is important to balance time considerations with costs, as environmental water screening usually involves numerous sites and frequent sampling.

**Acknowledgements**

This work was partially supported by Grant No. 01200622 from the National Science Foundation and Grant No. 1RO1A139129-01 from the National Institutes of Health.

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Detection, Isolation, and Identification of V. cholerae from the Environment

6A.5.36

Supplement 2

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