Published in final edited form as:
Curr Protoc Microbiol. 2012 August ; CHAPTER: Unit6A.5. doi:10.1002/9780471729259.mc06a05s26.

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

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Abstract

Recent molecular advances in microbiology have greatly improved the detection of bacterial pathogens in the environment. Improvement and a downward trend in the cost of molecular detection methods have contributed to increased frequency of detection of pathogenic microorganisms where traditional culture-based detection methods have failed. Culture methods also have been greatly improved and the confluence of the two suites of methods provides a powerful tool for detection, isolation, and characterization of pathogens. While molecular detection provides data on the presence and type of pathogens, culturing methods allow a researcher to preserve the organism of interest for "–omics" studies, such as genomic, metabolomic, secretomic, and transcriptomic analysis, which are rapidly becoming more affordable. This has yielded a clearer understanding of the ecology and epidemiology of microorganisms that cause disease. Specifically, important advances have been made over the past several years on isolation, detection, and identification of *Vibrio cholerae*, the causative agent of cholera in humans. In this unit, we present commonly accepted methods for isolation, detection, and characterization of *V. cholerae*, providing more extensive knowledge of the ecology and epidemiology of this organism. This unit has been fully revised and updated from the earlier unit (Huq, Grim et al. 2006) with the latest knowledge and additional information not previously

included. We have also taken into account of cost of reagents and equipment that may be prohibitive for many researchers and have, therefore, included protocols for all laboratories, including those with limited resources, likely to be located in regions of cholera endemicity.

Keywords

Vibrio cholerae; isolation; identification; detection; characterization

Unit 6A.4

Detection, Isolation, and Identification of *Vibrio cholerae* from Environmental Samples

It is well established that *Vibrio cholerae*, the causative agent of cholera, is autochthonous to the aquatic environment globally and is not confined to cholera endemic areas (Kenyon, Piexoto et al. 1984; Louis, Russek-Cohen et al. 2003; Schuster, Tyzik et al. 2011). Further, *V. cholerae* cells encoding genes and mobile elements known to cause disease in humans and confer resistance to antibiotics are found in the absence of cholera. When appropriate methods are used, *V. cholerae* can be detected in these aquatic environments throughout the year. *V. cholerae* culture negative water samples do not definitively indicate absence of this organism, as cells can enter into a viable but nonculturable (VBNC) state in which they may not form colonies on traditional bacteriological culture plates (Xu, Roberts et al. 1982; Roszak and Colwell 1987). In VBNC state, cell densities may be extremely low, therefore, if a small amount of water sample is collected, it may not contain a sufficient number of cells for detection. Thus, it is important appropriate volumes of water are examined, using appropriate methods to determine the presence of *V. cholerae* in a given sample.

This unit describes several widely accepted and highly cited methods used to detect, cultivate, enumerate, and characterize *V. cholerae* cells in environmental samples, such as water, sediment, plankton, and seafood, e.g., oysters. In developing countries, exposure to *V. cholerae* typically occurs via consumption of natural surface water containing *V. cholerae* in sufficient numbers to cause disease, whereas in developed countries exposure to *V. cholerae* often occurs via consumption of raw or undercooked shellfish or travel to regions where cholera outbreaks occur frequently. Recent outbreaks of shellfish related vibrioses and cholera in developed countries demonstrate the need to define a reliable method for detection of these organisms in any environmentally-derived food source (Onifade, Hutchinson et al. 2011). Both plankton and sediment play an important role in the occurrence and survival of *V. cholerae* in water and oysters. Sediment and plankton both can occur suspended within the water column. Sediment is known to harbor *V. cholerae* (due to its polar electrical charge). Plankton are known to be natural reservoirs of *V. cholerae* and zooplankton blooms facilitate increases in *V. cholerae* density. Furthermore, an infectious dose of *V. cholerae* (6×10^6 cells) (Cash, Music et al. 1974) can be present on a single plankton body (Huq, Small et al. 1984; Rawlings, Ruiz et al. 2007). Presence of *V. cholerae* in water, sediment, and plankton will also result in the presence of *V. cholerae* in shellfish, as these organisms filter feed all three through their bodies and concentrate high numbers of *V. cholerae*. It is, therefore, important to consider the type of sample and desired outcome (detection, isolation, or enumeration) when choosing the appropriate bacteriological/ molecular methods discussed here. For the readers' convenience, Table 1 and Figure 1 provide an overview of the methods provided in this unit.

STRATEGIC PLANNING

As stated above, the type of sample and the desired outcome are integral in determining which method(s) should be used to achieve satisfactory results. Furthermore, the skill set of

the laboratory members and laboratory resources are all essential in determining which tests can be conducted as all of these facets can vary dramatically from laboratory to laboratory as well as over time within one laboratory.

Traditional culturing methods, although yielding only a small subset of culturable bacteria in the original sample (< 0.1% of total bacteria), do allow researchers to archive individual strains and run a variety of informative bench top studies at a later date. These include genetic characterization by PCR, reconstruction of metabolic pathways by phenotypic analyses, gene expression assays, and whole genome sequencing, among many others. However, as stated earlier, reliance on traditional culturing methods only focuses one's scope on those bacteria which are culturable while inherently ignoring those bacteria cells which cannot grow on media but do remain a significant public health threat as they are capable of causing disease (Colwell 1991).

To evaluate the presence of these organisms regardless of their culturability, a direct detection method should be employed. Two microscopy methods are reliable, notably fluorescent antibody coupled with direct viable count (FA-DVC), or fluorescence in situ hybridization (FISH), a nucleic acid marker based method of detection. FISH can be utilized to detect genomic DNA sequences specific to *V. cholerae* regardless of serogroup, while FA-DVC can be used to detect *V. cholerae* of serogroups O1 and O139, serogroups associated with pandemic cholera. The direct fluorescence antibody (DFA) detection of serogroups O1 and O139 is more reliable than attempting to detect these strains via culture methods as targeting one serogroup in the environment is rather difficult to do, considering the vast serodiversity of *V. cholerae* in the environment. Both methods allow the researcher to quantify the target if no pre-enrichment step is used. Subsequent bacterial isolation is not possible when using this method unless a parallel sample is collected and processed using traditional culturing methods.

Direct molecular detection using the polymerase chain reaction (PCR) can also be employed to evaluate the presence of these organisms in environmental samples. These methods rely on detection of species-specific nucleotide sequences, followed by amplification of the targeted nucleic acid and visualization on an agarose gel (conventional PCR) or in an amplification plot using computer software (Real-Time PCR). Direct detection using these methods can be applied directly to environmental samples as well as to samples following a pre-enrichment step in alkaline peptone water (APW) or estuarine peptone water (EPW). All of these methods are highly sensitive, but it must be stated that absence of evidence of *V. cholerae* by a particular method does not confirm absence of this organism. As with all microbiological and molecular methods, inhibitors of the polymerase chain reaction, as well as quality and quantity of the collected sample and sensitivity of detection for each method must be taken into account when interpreting results. Replicate sample collection and replicate laboratory analyses further strengthen the statistical significance of results, but can dramatically increase level of effort as well as financial commitment.

Selection of sampling sites and specimen collection should be based on the goals of the study. It is valuable to select a variety of sites that are diverse in their ecology, with some sites selected with the *a priori* notion that they will harbor high densities of *V. cholerae* and others selected with the notion that they will harbor low densities of *V. cholerae*. If it is the goal to evaluate the presence or densities of *V. cholerae* in oysters then the overlying water column, underlying sediment, and planktonic organisms within the water column should also collected to discern the ecology of *V. cholerae* near the oyster beds of interest. During specimen collection, environmental parameters, such as, water temperature, salinity, conductivity, turbidity, and pH, should be recorded. For ecological investigations of organisms autochthonous to the aquatic environment it is necessary to collect and process

water, sediment, plankton, and, if possible, sea food (oyster) samples in a time series, preferably over a multi-year period with the aim to conduct sampling at regularly spaced intervals.

The collection of data over longer study periods allow researchers to estimate those parameters that may be associated with changes in *V. cholerae* densities or presence/absence in the selected area of interest. For studies utilizing these data to develop predictive models of *V. cholerae* in water bodies, readers are encouraged to review the reports of Louis, Russek-Cohen et al, 2003, Huq, Sack et al.2005, Constantin de Magny, Murtugudde et al. 2008, and Turner, Good et al.,2009, (Louis, Russek-Cohen et al. 2003), (Huq, Sack et al. 2005), (Constantin de Magny, Murtugudde et al. 2008) and (Turner, Good et al. 2009). In addition to consideration of time, resources, and logistics, the protocols in this unit allow a researcher the option to isolate and/or detect toxigenic *Vibrio cholerae* or total *Vibrio cholerae*. For all assays, media, materials, and methods should be validated with a positive control and a negative control. Several commonly used *V. cholerae* positive control strains that are typically available from culture collections, such as the American Type Culture Collection (ATCC), are listed in Table 2.

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.

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

Although developed several decades ago, traditional culturing methods continue to be improved with the result of more reliable detection of *V. cholerae* from environmental samples. The process involves either directly plating the study sample (water, plankton, sediment, shellfish) onto TCBS, TTGA, or CHROMAgar™ *Vibrio* or pre-enrichment of the sample in alkaline peptone water followed by streaking for isolation onto one or a combination of these three agars. For environmental samples, it is highly recommended to use pre-enrichment step to improve detection or isolation. Following an incubation period, presumptive *V. cholerae* can be isolated from these media and confirmed by either biochemical analyses (Huq, Grim et al. 2006) or immediately by PCR. Isolates can then be serogrouped as O1, O139, or non-O1/non-O139 by a slide agglutination assay using antisera for the O1 and O139 antigens or by PCR using primers developed to target O1 and O139 coding regions of the genomic DNA. These *V. cholerae* isolates can then be archived with replicates in nutrient agar containing 0.5% NaCl overlaid with sterile mineral oil or as a glycerol stock at −70°C. Stock cultures of *V. cholerae* should be periodically re-streaked for isolation to ensure their viability and purity.

BASIC PROTOCOL 1: Specimen Collection and Transportation

If the aim is to detect and isolate *V. cholerae* in water samples, then screening of concentrated water samples and plankton samples and “plankton-free” water is recommended, since a combination of all types of samples provides a higher probability of *V. cholerae* detection as well as a better understanding of the role of plankton in *V. cholerae* dynamics in the study region (Huq, Colwell et al. 1990; Binsztein, Costagliola et al. 2004; Huq, Sack et al. 2005; Turner, Good et al. 2009; Lizarraga-Partida, Wong-Chang et al. In preparation). Water collection bottles should be cleaned with detergent, however the latter **must not** leave residue, should not be anti-bacterial and they should be pre-sterilized in an autoclave for 15 to 20 minutes at 121°C prior to use. Polypropylene bottles should be used for water samples and glass bottles should be used for plankton samples. A sufficient

volume of water and plankton should be collected to insure that appropriate analyses can be performed. Plankton samples should be collected by passing water through a 200, 64, and/or 20-μm pore size plankton net by towing the net manually or behind a boat. Alternatively, known volume of water can be manually passed through the net by bucket pouring or pumping, which can be useful for quantitative analysis (Huq, Sack et al. 2005).

Processing of samples should begin soon after collection (typically within 24 hours of collection). If enumeration of *V. cholerae* is desired, then the sample should be stored in a cool box at a temperature of 10 to 15°C until processing begins (not to exceed 8 hours) (Clesceri, Greenberg et al. 1998). However, a recent study has demonstrated that transporting samples at ambient air temperature prior to processing can enhance the culturability of *V. cholerae* in the sample (Alam, Sadique et al. 2005). Based on type of examination, samples may require treatments; such as addition of direct viable count (DVC) reagents, before proceeding with further examination and testing. It is recommended that basic physiochemical parameters, e.g., temperature, salinity, pH, dissolved oxygen and conductivity of the water be measured on site at the time of collection as it is known that *V. cholerae* densities can be influenced by such parameters. If the study site is influenced by tides, coasts and estuaries for example, the prevailing tidal level at the time of sampling should be recorded. Furthermore, in addition to water temperature, significant correlation of water depth, rainfall, conductivity, and copepod density with cholera outbreaks has been reported, with lag periods from zero to eight weeks from optimum environmental conditions to cholera outbreaks (Huq, Sack et al. 2005). These parameters can be measured on site using portable meters (such as HACH Model CO150 Conductivity meter, HACH Chemical Company, Loveland, CO or Dissolved oxygen and pH meter, Model 210A, Orion Laboratories, Beverly, MA). It is important to note that in all studies of the ecology of microorganisms in the environment several rounds of “practice” sampling and processing should be considered to optimize the volumes of water, plankton, or other biotic and abiotic media to be collected in order to detect or recover the specific microorganism of interest, as well as to identify missing equipment and reagents and evaluate the ability to comprehend and adhere to the standard methods described herein. It is hard to keep the sample collection volumes constant as the amounts (volumes) may need to be adjusted depending on the quality of water due to presence of seasonal plankton. The authors of this unit strongly encourage investigators to use several methods and several types of media throughout the duration of their studies. All methods have some error or detection limits associated with them and, therefore, use of multiple approaches will allow the investigators to converge on the most accurate answer. Water, sediment, and oysters isolated from different environments, both on a global and local scale, may have different chemistry associated with them and therefore, the different media requirement discussed in this unit may perform more or less effectively over time and space. Diversifying one’s approach to gathering data on the ecology of *V. cholerae* will help overcome these challenges. The authors also encourage the investigators to use a range of incubation temperatures for APW enrichment and growth on selective agar plates. It is well known that *V. cholerae* can grow within a wide range of temperatures and that this group of organisms is phenotypically heterogeneous. Therefore, utilizing two incubation temperatures may increase the probability that *V. cholerae* is detected and/or isolated. It should be known that this may effectively double the amount of work downstream, but the investigators should also be aware that several methods of isolation and detection will allow convergence on an accurate analysis.

Materials

Pre-sterilized 500-ml glass (Qorpak, Bridgeville, PA) and 1000-ml polypropylene containers (Nalgene, Rochester, NY)

Simple Plankton nets, 200-μm, 64-μm, and 20- μm, or nets of different mesh sizes (for size filtration of plankton) (Aquatic Research Instruments, Idaho, USA; SEA-GEAR, Florida, USA) (See Figure 2).

Portable meter(s) that measure temperature, dissolved oxygen, pH, turbidity and salinity (HACH Company, Colorado, USA; Geo Scientific Ltd, British Columbia, Canada; YSI Inc. / Xylem Inc., Ohio, USA). Turbidity and salinity can be measured *ex situ*, i.e. in the laboratory, preferably immediately after sample collection.

Bucket of known volume–e.g., 4 or 5 liters (optionally used for pouring water through plankton nets). ≥ 92-oz. Whirl-Pak® Bags (Nasco, Fort Atkinson, WI)

Autoclaved and pre-weighed 100-ml polypropylene container (optionally used when collecting sediment from an oyster bed)

Scooper/Spoon that has been wrapped in aluminum foil and autoclaved (optionally used when collecting sediment from an oyster bed)

To collect water sample

1. Uncap pre-sterilized plastic bottle, submerge bottle to fill. Fill to half of volume of bottle, re-cap, shake to rinse and discard. Repeat 3 to 5 times. Rinse downstream of water sample collection site if possible.
2. Remove the sample bottle from water. Cap, leaving enough air in the bottle for agitation and mixing.

To collect plankton

3. Rinse plankton net and cod-end of the net in the body of water to be sampled (downstream of sample collection site if possible).
4. Filter 10 to 100 liters water through the plankton net by towing, using a calibrated, net-mounted flow meter, or pouring known-volumes through the net with a smaller bucket (the bucket should be clean, but without detergent, and rinsed several times with sample water, downstream of the sample collection site, immediately prior to sample collection).
5. For each plankton fraction of interest, rinse inside the net of the plankton net followed by the cod-end with sterile 1X PBS using a spray or squirt bottle, or with excess plankton-free water collected after pouring sample water through the net. This allows any plankton adhering to the net to be flushed down to the cod-end for collection.
6. Remove cod-end from the plankton net and decrease volume to 100 ml by continuing to filter. This can be done by gently swirling the cod end so that water passes through the mesh siding of that cod end.
7. Measure (with a sterile graduated cylinder) and decant plankton fraction into sterile glass container making sure to label that bottle with the collected plankton fraction size.
8. Repeat this process for all plankton fractions of interest.
9. Finally, collect 500 ml to 1000 ml water that passes through the 20-μm plankton net. This is “plankton-free” water.

To collect Sediment Sample

1. Collect sediment with a piston corer (Aquatic Research Instruments, Hope, ID) or a Peterson grab (Wildlife Supply Company, Yulee, FL).
2. Sample a sediment–water interface with a wide-bore serological pipette and put in a sterile bottle (this sediment-water interface sample can be treated as a separate sediment sample and processed alongside the other media collected following the same protocols).
3. Remove the remaining sediment from the sampling apparatus aseptically and put into a sterile bottle.

To Collect Shellfish—*Shellfish collection in many areas is regulated by local or state governments. It is imperative to check these regulations with your local Fish and Wildlife department or other relevant government bodies prior to sample collection. Collection of shellfish may require purchase of a permit and in some locations, may be done only on certain days. The oyster processing protocols described here can be used to evaluate store bought oysters. However, these oysters may have been depurated and stored for some time and results from these analyses should be described with respect to post-harvesting conditions, not the environment from which these oysters were harvested. Additionally, since oyster shells may be sharp, it is advised to wear gloves whenever handling oysters in order to avoid injury.*

1. Collect oysters from boats by using oyster tongs or a dredge or hand collect at low tide when the oyster beds are easily accessible.
2. Examine oysters should for viability in the field by keeping only those oysters that are tightly closed. Tapping the mid-shell point of an oyster on a hard surface will allow determination whether oyster meat is present in the oyster shell because a hollow sound will be heard when an empty oyster is tapped. Rinse sediment off of the oyster shells with surface water, but do this only after water and plankton samples have been collected.
3. Place viable oysters in ventilated ≥ 92-oz. Whirl-Pak® Bags (Nasco, Fort Atkinson, WI) and transport in a cool container, but not immediately next to ice or cool packs. Do not re-immerse oysters in water after collection.

To Collect Sediment from an Oyster Bed—*If oysters are already being collected during sample collection, it is possible to collect sediment samples at the same time in order to avoid using a piston corer as direct sediment sample collection from a hard oyster bed (also known as an oyster reef) may be difficult.*

1. When determining whether or not oysters are present in the shell by tapping, hollow oyster shells may contain sediment inside; therefore it is imperative to open the oyster shells to check for sediment.
2. If sediment is found, use a scooper that has been previously wrapped in aluminum foil and autoclaved to scrape the sediment off the shell. Place sediment in a sterile, pre-weighed 100-ml polypropylene container.

Transportation and/or processing

Transport samples to the laboratory for processing or, preferably, begin processing onsite within 1 hr of collection.

For samples that require transport to the laboratory to be processed after collection, store and/or transport the samples in a cold box at 10° to 15°C. Alternatively, samples can be kept

in the dark at ambient air temperature (22° to 25°C) after collection for up to 24 hours as this may enhance recovery of Vibrio species as shown in a recent study (Alam, Hasan et al.) and can be a useful approach for detecting vibrios in those environments where the organism is predominantly in the viable but non-culturable (VBNC) state (Alam, Hasan et al. 2006). This aspect of the protocol may need to be optimized for the water source and environmental conditions.

BASIC PROTOCOL 2: 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 series of biochemical tests or PCR and serological tests to determine strain serotype. Alternatively, biochemical tests can be bypassed and confirmation can be done using PCR if adequate supplies of PCR reagents are available. Most water and plankton samples require some amount of concentration. Running several practice rounds at the desired study site will help estimate appropriate volumes of sample that should processed in order to achieve desired goals. However, it should be pointed out that environmental *V. cholerae* densities are not static, so volumes deemed to be appropriate at one sampling point may not yield the same results at another time. We address this inconsistency by urging the investigator to use multiple methods (if resources are available) to isolate, detect, and characterize environmental *V. cholerae* during the course of their study. Water samples, both whole water and plankton-free water should be concentrated by filtration using 0.2-μm polycarbonate membrane filters. A good starting volume is 500-ml and 1000 ml can be used if water passes easily through the polycarbonate membrane. If 0.2-μm polycarbonate membrane filter clog quickly, multiple filters can be used per sample. It is preferable to use 0.2-μm pore size filters as opposed to 0.45-μm pore-size filters as cells VBNC bacteria are often < 0.45-μm in size. Polycarbonate membranes are preferable to nitrocellulose membranes as the cells sit on top of the polycarbonate filters and are easily removed by vortexing while cells get caught in the fibers of nitrocellulose membranes. It is imperative that the cells be physically agitated off of the membrane so that they may migrate to the microaerophilic layer of the enrichment broth discussed below.

Overnight enrichment is performed using alkaline peptone water (APW), pH 8.6 and some investigators recommend two successive enrichments. Surface aliquots from the microaerophilic pellicle layer are streaked for isolation onto selective bacteriological media. Three commonly used selective media for *V. cholerae* isolation are thiosulfate citrate bile-salts sucrose (TCBS) agar, tellurite taurocholate gelatin agar (TTGA), also known as Monsur medium (Monsur 1961), and CHROMagar™ *Vibrio* (CHROMagar, Paris, France). *V. cholerae* appears as translucent, flat, yellow colonies with elevated centers on TCBS and colorless colonies on TTGA, often with a characteristic dark center after two days growth, surrounded by a halo, which appears due to the hydrolysis of gelatin, and turquoise colonies on CHROMagar™ *Vibrio* (Fig. 3). If possible, use more than one medium as that may allow convergence on the best results. APW enriches for many bacteria other than *V. cholerae*, therefore, direct plating of samples onto TCBS, TTGA, and/or CHROMagar™ *Vibrio* can be done in parallel with APW enrichment and may yield *V. cholerae* colonies that can be used for further analyses. This direct plating method is beneficial in instances when overgrowth of non-target bacteria occurs on solid media after overgrowth in APW.

TCBS is a highly selective medium that may, in rare instances, inhibit growth of *V. cholerae* and at the same time allow growth of non-*Vibrio* organisms, such as *Aeromonas sp.*, *Staphylococcus sp.*, and *Shewanella sp.* TCBS is commercially available and produced by several companies which have global distribution. TTGA media is considered to be less inhibitory to *V. cholerae* cells; however longer incubation times are needed (48 hours) to observe the dark center that is characteristic of *V. cholerae* on this medium. It may be

difficult to distinguish *V. cholerae* from *V. parahaemolyticus* on this medium, therefore, modified TTGA media can be used in which *V. cholerae* colonies appear as brilliant blue with fluorescence in 24 hour or less, by adding β-galactosidase (4-methylumbelliferyl-β-Δ-galactosidase) (O'Brien and Colwell 1985). CHROMagar™ *Vibrio*, primarily used for the detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*, distinguishes these organisms from each other based on a proprietary chromogenic reaction that prevents making it in the laboratory from scratch. Both TCBS and CHROMagar™ *Vibrio* need not be autoclaved and incubation times for these media are shorter (24 hours) than TTGA. TTGA media must be autoclaved prior to use. Once presumptive strains are purified on a nonselective medium, such as gelatin agar, modified nutrient agar, or Luria Bertani (LB) agar with 1% NaCl, they are confirmed and serogrouped by PCR or by simple slide agglutination, employing polyclonal *V. cholerae* O1, O139 and monoclonal Inaba and Ogawa antiserum.

Materials

Tissue homogenizer (hand held) to homogenize plankton (Kimble Chase Life Science and Research Products LLC, New Jersey, USA)

Filter apparatus with vacuum source

Filter membranes, 47-mm diameter, 0.22-μm pore size

Forceps

Enrichment flask (sterile 150-ml Erlenmeyer flasks containing 25-ml APW)

Alkaline peptone water (10× and 1× APW), pH 8.6

Phosphate buffered solution (PBS), pH 7.4

Thiosulfate citrate bile-salts sucrose (TCBS) agar plates

Tellurite taurocholate gelatin agar (TTGA) plates

CHROMagar™ *Vibrio* (written as “CA” in the following sections)

Gelatin agar (GA), modified nutrient agar, or Luria-Bertani agar (LB) plates

Inoculating loops and needles

Incubators, 30 to 37°C

Sterile Petri dishes

Cut-resistant oyster shucking glove (Chef Revival, WI, USA)

Sterile oyster knife (Dexter-Russell, Inc., MA, USA)

Sterile toothpicks

Oxidase reagent: 1% (w/v) *N,N,N’N’*-Tetramethyl-*p*-phenylenediamine dihydrochloride 95% alcohol for flame sterilization of forceps, loops, and needle, bacterial cell spreader.

Sample Processing—*Note: If not specified, assume APW is 1X APW.*

1a. *For plankton samples:* Filter concentrated plankton sample through a 47-mm, 20-μm pore size polycarbonate filter (Millipore, Billerica, MA) (this step may take a while depending on the abundance of plankton in the sampled water). Add the filter(s) with attached bacteria to a 50 ml centrifuge tube with 25 ml of sterile 1X PBS.

Vortex the centrifuge tube in order to remove cells from the filter.

Add 1-ml whole plankton to 25-ml APW.

Add 100 to 1000 μl of whole plankton directly onto TCBS and/or CA and TTGA plates and spread using a bacterial cell spreader.

Homogenize 10-ml of concentrated plankton samples using a glass tissue grinder by moving the pestle up and down in the tube, while rotating, 10–20 times. Add 1-ml homogenized plankton to an enrichment flask containing 25-ml APW.

Add 100 to 1000 μl of homogenized plankton directly onto TCBS and/or CA and TTGA plates and spread using a bacterial cell spreader.

1b. *For water samples:* Filter 100 to 1000 ml (depending on bacterial density, see comment at end of "Specimen Collection and Transportation") water through a 47-mm, 0.22-μm pore size polycarbonate filter. Add the filter(s) with attached bacteria to a 50 ml centrifuge tube with 12 ml of sterile 1X PBS. Vortex vigorously for ca. 5 minutes to detach the bacteria from the filter.

Add 100 to 1000 μl of this 1X PBS containing detached bacterial cells directly onto TCBS and/or CA and TTGA plates and spread until plates appear dry using a bacterial cell spreader.

Add 1-ml 1X PBS containing detached bacterial cells to an enrichment flask containing 25-ml APW.

This step may be done in replicate to increase the probability of isolating V. cholerae. However, it should be known that replication at this step dramatically increases the magnitude of analyses downstream.

Large volumes or highly turbid sample water may require more than one polycarbonate filter, as they will clog.

The remaining 1X PBS containing detached bacterial cells can be stored at −20°C for other direct molecular detection methods including metagenomic analysis.

1c. *For oyster samples:* Rinse and scrub exterior of oysters in sterile de-ionized water making sure to remove sediment from the oyster hinge. With extreme caution, wearing a cut-resistant oyster shucking glove, shuck oysters with a sterile oyster knife. It may help to also hold the oysters level on a table with a towel between your glove and the oyster. This may add protection and stability. When shucking, with the curved-side of the knife facing downwards, trace the knife along the upper shell making sure to sever the adductor muscle which holds the oyster shells shut. Shuck enough oysters to produce 250 g of oyster meat as well as liquid surrounding the oyster meat and add to an autoclavable kitchen blender. Add an equal volume of sterile 1X PBS and homogenize for 90 seconds.

Add 20 ml of homogenized oyster sample to an enrichment flask containing 80mL of 10× APW.

Add 100 to 1000 μl of oyster homogenate to TCBS and/or CA and TTGA plates and spread using a bacterial cell spreader. This step may be further adjusted to include both direct plating of 100 to 1000 μl onto agar as well as plating serial dilutions of homogenized oyster meat in sterile 1X PBS.

If an autoclavable blender is not available the interior of the blender pitcher can be sterilized with 10% bleach or 70% alcohol followed by multiple rinses with sterile DI

water or 1X PBS. It is imperative that all bleach or alcohol be removed prior to adding the oyster meat. Alternatively, a stomacher could be used.

To sterilize the oyster knife, dip blade into 95% ethanol and then pass through a flame. If oyster knife is autoclavable it can be wrapped in aluminum foil and sterilized at 121°C for 15 minutes. Oysters can be shucked when blade is cool.

1d. *For sediment samples:* Weigh sediment samples and add an equal volume:weight of sterile 1X PBS and vortex or shake well. Add 100-ml sediment/PBS slurry to an enrichment flask containing 11 ml 10X APW.

Additionally add 100 to 1000-µl sediment/PBS mixture directly onto TCBS and/or CA and TTGA plates and spread using a bacterial cell spreader. This step may be further adjusted to include both direct plating of 100 to 1000 µl onto agar as well as plating serial dilutions of sediment in sterile 1X PBS.

2. Incubate the APW enrichment flasks statically for 16 hr at 30 to 35°C (overnight).

In all cases, APW enrichment flasks should not be disturbed or agitated during or after incubation, as Vibrio species tend to migrate to the liquid-air interface.

A range of incubation temperatures can be used as V. cholerae is known to grow between 12 and 42°C. Lower incubation temperatures for longer periods of time may allow for more V. cholerae cells to grow while also allowing for other competitive bacteria to grow as well. Higher incubation temperatures may inhibit growth of some environmentally-stressed V. cholerae cells but will also inhibit growth of competitive bacteria species. It may be beneficial to consider testing two incubation temperatures and choosing which yields better results for your sample area of interest. Alternatively, replicate flasks can be incubated at each temperature to increase the probability of isolating V. cholerae (see italicized note immediately after section 1b in the Sample Processing section).

3. Incubate TCBS, CA at 30 to 35°C for 24 ± 2 hours and TTGA plates at 30 to 35°C for 48 hours.

The same approach as stated above for incubation temperatures of APW inoculates can be applied, and should be considered for incubation of agar plates.

Subculture smooth, flat, sucrose-fermenting, yellow colonies from TCBS and/or turquoise blue colonies from CA, and translucent, dark-centered colonies with halo zones from TTGA (see Figure 3) onto GA and/or modified nutrient agar plates.

Selective plating post-APW enrichment

3. After enrichment in APW, collect surface growth (may be present as a whitish film) from the enrichment flask with an inoculating loop and streak onto TCBS and/or CA and TTGA plates.

4. Incubate the plates 16 to 24 hr at 30 to 35°C for TCBS and CA or 48 hr at 30 to 35°C for TTGA.

5. Subculture smooth, flat, sucrose-fermenting, yellow colonies from TCBS and/or turquoise blue colonies from CA, and translucent, dark-centered colonies with halo zones from TTGA (see Figure 3) onto GA and/or modified nutrient agar plates, respectively.

To subculture presumptive V. cholerae colonies from these selective media, touch the top-center of the colony using a sterile inoculating loop. Take care not to touch the

surface of the agar and any other surrounding bacteria (even if they too are presumptive V. cholerae). If plates are heavily overgrown with bacteria, try to touch the top-center of a presumptive V. cholerae colony and re-streak onto selective media again to isolate a single colony.

If TCBS is used, morphology should be registered only on recent and well-isolated colonies.

ALTERNATE PROTOCOL 1: Bacteriological Culture Method for Situations of Limited Resources

This alternative protocol can be used to presumptively estimate the presence of culturable *V. cholerae* in surface waters in regions where even general microbiology resources are limited. Bacteriological methods are based on the work of Choopun et al.(Choopun, Louis et al. 2002). Yellow colonies isolated from TCBS that give negative reactions (no color change) in esculin hydrolysis and arginine dihydrolase assays can be presumptively identified as *V. cholerae.*

Glassware can be used for assays and can be sterilized by wrapping in aluminum foil or newspaper and then heated in an oven at 180°C for 2 hours. Sterilization can also be accomplished by microwaving materials for 5 minutes. Investigators must make sure that no non-microwavable materials are used (i.e., aluminum foil). Mineral oil should be sterilized by heating in an oven at 170°C for 1 to 2 hours. If it is not possible to procure autoclavable sample collection bottles then store bought drinking water bottles may be used. It is important to rinse these bottles with surface water from the collection site 3 to 5 times prior to collecting the sample for analysis.

Materials

1 Liter Plastic or glass water sample collection bottles

Enrichment bottle (1 Liter glass bottles)

Aluminum foil

Newspaper or other paper source

Conventional oven or microwave

Incubators, 30 to 37°C

Inoculating loops and needles

Sterile petri dishes

Sterile toothpicks

Sterile test tubes capped with paper, cotton, or aluminum foil prior to sterilization

Alkaline peptone water (10× and 1× APW), pH 8.6

Thiosulfate citrate bile-salts sucrose (TCBS) agar plates

Selective media for *V. cholerae* isolation (TTGA, TCBS, or CA)

Gelatin agar (GA), modified nutrient agar, or Luria-Bertani agar (LB) plates

Heart infusion agar containing 0.1% esculin and 0.05% ferric chloride.

Luria-Bertani broth containing 1% (wt/vol) L-arginine (pH 6.8) and phenol red powder added as an indicator.

Sterile mineral oil

1. Add 100-ml to a sterile bottle containing 900-ml 10X APW and shake or swirl well.
2. Incubate at 30 to 35°C overnight.
3. With a sterile loop or toothpick dab the upper pellicle layer of the incubated sample and streak for isolation onto a selective medium for *V. cholerae* isolation (TTGA, TCBS, or CA). Incubate plates at 30 to 35°C overnight (or 48 hours if TTGA agar is used).

If incubators are not available then 10X APW inoculation can be left to sit at room temperature for 24 hours and agar plates can be inverted (agar-side up) and left to sit in the dark until growth occurs (up to 2 days).

4. For esculin hydrolysis assay gently touch the top and center of a yellow non-swarming colony with a sterile loop or toothpick and inoculate into heart infusion agar containing 0.1% esculin and 0.05% ferric chloride.
5. For arginine dihydrolase assay simultaneously inoculate Luria-Bertani broth containing 1% (wt/vol) L-arginine (pH 6.8) and phenol red powder (Difco) added as an indicator. After inoculation cover with ca. 0.5 cm sterile mineral oil.
6. Incubate esculin hydrolysis assays at 30 to 35°C for 72 hours or at room temperature for approximately 96 hours and arginine dihydrolase assays for 24 hours at 35°C or 48 to 72 hours at room temperature.

Blackening of the esculin hydrolysis medium after incubation indicates that that there was a positive reaction while appearance of a red color after incubation of the arginine dihydrolase assay was considered a positive reaction. For both assays, a negative control (uninoculated assays) should be incubated at the same temperature for the same time as a comparator. Used materials, including inoculated media, can be sterilized by microwaving for 5 minutes. Used materials can also be sterilized in a dry oven at 180°C for 2 to 3 hours.

BASIC PROTOCOL 3: Serogroup Determination

More than 200 serogroups of *V. cholerae* have been described to date based on antigenic property of cell surface polysaccharides of which, serogroup O1 and O139 have been implicated with epidemics of cholera while non-O1/non-O139 serogroups are known to cause sporadic outbreaks. However, serogroup O37 was responsible for a localized outbreak of cholera in Czechoslovakia and Sudan. The remaining serogroups collectively and commonly termed as non-O1/non-O139, predominate the strains of *V. cholerae* isolated from the aquatic environment (Sack, Siddique et al. 2003). Although reported mostly in O1 and O139 serogroup clinical isolates, the cholera toxin gene can be found in non-O1/non-O139 strains from the aquatic environment globally as well as other *Vibrio* species (Chakraborty, Mukhopadhyay et al. 2000; Malayil, Turner et al. 2010). However, because of the epidemic potential, the method to determine O1 and O139 serogroup is mentioned 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

Antiserum for serogroup O1 and O139 *V. cholerae*

Glass slides

Wax pencils

Phosphate buffered saline (PBS)

1. Draw 2 squares approximately 6 cm × 6 cm on a microscope slide with a wax pencil. Add one drop of PBS in each square.
2. Add a loopful of fresh growth (6–16 hr subculture on non-selective media) into each drop and resuspend.
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.

   Agglutination can be better visualized by looking through microscope slide with a bright lamp in the near background.

   Autoagglutination, clumping in the saline solution without antiserum, is indicative of a "rough" morphotype and cannot be typed by antisera.
5. Test non-O1 serogroup colonies with O139 antisera, following steps 1–4 (Basic Protocol 3) using O139 antisera.

MOLECULAR METHODS FOR DETECTION AND IDENTIFICATION OF *V. CHOLERAE ISOLATES*

Conventional PCR and Real-Time PCR have recently been used to characterize *V. cholerae* strains, along with confirming a strain as *V. cholerae* or confirming the presence of *V. cholerae* in environmental or clinical samples. Further, the increase in genome sequences now available has allowed development of PCR-based typing schemes for analysis to variants of strains, genes, and mobile genetic elements, including pathogenicity islands. The body of knowledge derived from the *V. cholerae* pangenome continues to provide researchers with targets useful in strain identification and characterization.

Identification and characterization of Suspected or Presumptive *V. cholerae* Isolates by PCR

The polymerase chain reaction (CPMB 15.1) is a useful alternative to labor-intensive biochemical tests, which are occasionally difficult to interpret, and often requiring replication, as well as several days of incubation and media preparation. Ideally, at least an oxidase test (Basic Protocol 2) should be done on presumptive colonies to reduce the number of presumptive *V. cholerae* isolates that are not *V. cholerae*. In this method, crude template is prepared by boiling to lyse the cells. Genomic regions within this template are amplified using PCR pimers specific to *V. cholerae* and targeting the internal transcribed spacer (ITS) region between 16S and 23S rDNA or the outer membrane protein subunit W (*ompW*). Confirmed *V. cholerae* strains can be screened for the genes associated with virulence and their variants (Table 3) and a more comprehensive list of relevant targets, with PCR conditions and expected amplicons, is listed in Supplementary Table 1. 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 eubacterial 16S rDNA PCR reaction for each sample to test template quality. (See Table 3 for PCR primers, expected amplicon size and reference). Further, phenotypic and genetic screens on *V. cholerae* isolates can be done following the methods of Son and Taylor (Son and Taylor, 2011).

SUPPORT PROTOCOL 1: Preparation of crude DNA template by boiling

A crude DNA template, suitable for analysis in Basic Protocol 4, Alternate Protocol 2, and Basic Protocol 5, can be prepared by boiling a small overnight culture or a loopful on culture from a fresh agar plate.

Materials

1-ml overnight culture or loopful of pure culture from agar plate

1a. *From broth*: Centrifuge a 1-ml culture (overnight growth at 35°C) and resuspend in 1-ml sterile water.

1b. *From agar plates:* Resuspend a loopful of pure culture (50–100 colonies) of suspected or presumptive *V. cholerae* into 300-µl of sterile water by vigorously vortexing. *Plates should be a fresh overnight subculturing demonstrating only one morphology.*

2. In a sterile 2-ml microcentrifuge tube, dilute the suspension 1:1000 in sterile water.

Alternately, a single isolated colony can be resuspended into 20-µl of sterile water.

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 (approximately 30 minutes).

Treatment of crude DNA template with 10-mg/mL bovine serum albumin (BSA) (4-µl per 100-µl supernatant) may help to limit PCR inhibition and yield more accurate results. It is also beneficial to make a 1:10 dilution and a 1:1000 dilution (supernatant:sterile DI water) and use this for PCR as well as a dilution may also help to remove PCR inhibitors.

BASIC PROTOCOL 4: Vibrio cholerae-specific PCR - ITS (Chun, Huq et al. 1999)

The following PCR methods can be used to confirm isolates as *V. cholerae* by targeting the internal transcribed spacer (ITS) region between 16S and 23S rDNA or the outer membrane protein subunit W (*ompW*) specific to *V. cholerae*.

Materials

20 µM PCR primers (Table 3)

25 µm dNTPs (*APPENDIX 2A*)*

Taq DNA polymerase

PCR amplification buffer, 10X (*APPENDIX 2A*)

Sterile water

Molecular weight ladder (e.g. Hyperladder IV, Bioline, Taunton, MA)

Thermal cycler (BioRad, Hercules, CA)

Microcentrifuge tubes

Boiling water bath

PCR tubes

Horizontal gel apparatus, gel tray and comb.

Power supply for gel apparatus

1 μg/ml ethidium bromide staining solution (*APPENDIX 2A*)

UV transilluminator (UVP, Upland, CA)

*"All-in-one" PCR mastermix can be purchased as an alternative to buying the necessary reagents separately. This can facilitate screening large numbers of strains. PCR primers and nuclease-free water will need to be purchased separately. Several products include, but are not limited, to GoTaq® Green Master Mix (Promega, Madison, WI) and Multiplex PCR 5X Master Mix(New England BioLabs, Ipswich, MA) for detecting multiple targets by PCR.

*Alternative DNA staining dyes that may be less mutagenic have recently been developed and can be explored as an alternative to ethidium bromide. Dyes include GelRed™ and GelGreen™ (Biotium, Hayward, CA), SYBR® Safe DNA Gel Stain (Life Technologies, Grand Island, NY). Gels stained with GelGreen™ can be visualized with a laser-based gel scanner or a Dark Reader that uses visible blue light for excitation. Gels stained with SYBR® Safe DNA Gel Stain can be visualized under blue light as well.

5. Set up *V. cholerae*- specific ITS (Internal Transcribed Spacer region) PCR in a total reaction volume of 25-μl, containing the following.

5 μl template (step 4)

1X reaction buffer

200 μM dNTPs

800-nM primers (pVC-F2, pVCM-R1)

0.625 U *Taq* DNA polymerase.

6. Amplify the *V. cholerae*-specific ITS target with the following cycle conditions:

Initial denaturation :	1 min at 94°C (initial denaturation)
30 cycles:	1 min at 94°C (denaturation)
	1 min at 60°C (annealing)
	1 min at 72°C (extension)
Final extension:	10 min at 72°C.

Perform Agarose Gel Electrophoresis and Visualize Results

7. Run PCR product out on a 1.5% agarose gel in 1X TAE for 1–2 hrs at 5-V/cm (CPMB 2.5A).

8. 8. Stain the gel in 1 μg/ml ethidium bromide staining solution for 15 min.

9. Destain the gel in distilled water for 15 min.

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

The V. cholerae 16S-23S rDNA intergenic spacer region amplicon is 300-bp in size (Fig 4, lane 2).

11. Screen ITS-PCR confirmed *V. cholerae* isolates for the virulence-associated factors listed in Table 3 and/or Supplementary Table 1. See Figure 4.

* Using GoTaq® Green Master Mix (Promega, Madison, WI), prepare the PCR reaction as follows for a total reaction volume of 25 µL; 5µl template DNA (step 4, 12.5 µl 2× GoTaq® Master Mix (containing GoTaq® DNA Polymerase, 400µM dATP, 400µM dGTP, 400 µM dCTP, 400 µM dTTP, 2mM $MgCl_2$), 1µl of each primer (20 µM pVC-F2 and pVCM-R1), 5.5 µl nuclease free water

ALTERNATE PROTOCOL 2: Multiplex PCR assay for detection of ompW (V. cholerae-specific) and ctxA (toxigenicity) (Nandi, Nandy et al. 2000)

This protocol can be used in place of Basic Protocol 4 since it detects for the *V. cholerae*-specific *ompW* sequence. It also allows for the detection of the *ctxA* gene of the cholera toxin.

Materials

Crude DNA template (Support Protocol 1) or extracted genomic DNA (Basic Protocol 10)

Reaction buffer

250 uM dNTPs

Omp W F and R primers

ctxA F and R primers

Taq polymerase

1.5% agarose gel

TAE buffer

Ethidium bromide staining solution

5. Set up *ompW-ctxA* multiplex PCR in a total reaction volume of 25-µl, containing the following:

10 to 20 ng of crude DNA template (Basic Protocol 4, step 4) or extracted genomic DNA (Basic Protocol 11, *DNA extraction using phenol, chloroform, and isoamyl alcohol*, step 13)

1X reaction buffer

250-µM dNTPs

1.2-pmol/µl of *ompW* (ompW-F, R) primers

0.25-pmol/µl *ctxA* (ctxA-F, -R) primers

0.625-U Taq polymerase.

6. Amplify the targets with the following cycle conditions:

Initial denaturation:	5 min at 94°C
30 cycles:	30 sec at 94°C (denaturation)
	30 sec at 64°C (annealing)
	30 sec at 72°C (extension)
Final extension	7 min at 72°C.

7. Run PCR product out on a 1.5% agarose gel in 1X TAE for 1–2 hrs at 5-V/cm (CPMB 2.5A).

8. Stain the gel 15 min in 1 µg/ml ethidium bromide staining solution.

9. Destain the gel 15 min in distilled water.

10. Visualize the products by viewing the gel under UV light.

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

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

BASIC PROTOCOL 5: Multiplex PCR assay for detection of O1 and O139 serogroup *V. cholerae* and *ctxA* (Hoshino, Yamasaki et al. 1998)

The multiplex PCR assay is performed to confirm O1 and O139 somatic antigens and for the simultaneous detection of the α-subunit of the cholera toxin gene sequence, *ctxA* in confirmed *V. cholerae* isolates.

Materials

20 μM PCR primers (Table 3)

25 μm dNTPs (*APPENDIX 2A*)*

Taq DNA polymerase

PCR amplification buffer, 10X (*APPENDIX 2A*)

Sterile water

Molecular weight ladder (e.g. Hyperladder IV, Bioline, Taunton, MA)

Thermal cycler (BioRad, Hercules, CA)

Microcentrifuge tubes

Boiling water bath

PCR tubes

Horizontal gel apparatus, gel tray and comb.

Power supply for gel apparatus

1 μg/ml ethidium bromide staining solution (*APPENDIX 2A*)

UV transilluminator (UVP, Upland, CA)

1. Set up O1/O139-*rfb/ctxA* multiplex PCR in a total reaction volume of 30-μl, containing the following:

10 to 20 ng of crude DNA template (Support Protocol 1, step 4) or extracted genomic DNA (Basic Protocol 10, step 13)

1X reaction buffer

210 μM dNTPs

0.5 μM O1-*rfb* (O1F2-1, O1R2-2) primers

0.27 μM O139-*rfb* (O139F2, O139R2) primers

0.17-μM *ctxA* (VCT1, VCT2) primers

0.75-U Taq polymerase.

2. Amplify the targets with the following cycle conditions:

Initial denaturation:	5 min at 94°C
35 cycles:	1 min at 94°C (denaturation)
	1 min at 55°C (annealing)
	1 min at 72°C (extension)
Final extension:	7 min at 72°C.

3. Run PCR product out on a 2.0% agarose gel in 1X TAE for 1–2 hrs at 5-V/cm (CPMB 2.5A).
4. Stain the gel 15 min in 1 μg/ml ethidium bromide staining solution.
5. Destain the gel 15 min in distilled water.
6. Visualize the products by viewing the gel under UV light.

The O1-rfb, O139-rfb, and ctxA amplicons are 192, 449, and 308-bp in length, respectively. (See Fig 4)

Real-Time PCR for Detection of *V. cholerae*—DNA-based methods such as PCR increasingly have been employed. These are designed for rapid, sensitive analysis of a range of clinical and environmental samples. End point PCR is not quantitative and the presence of the PCR product(s) must be verified using a procedure such as southern hybridization and gel electrophoresis (Lyon 2001).

It is important to note that, as in the case of conventional PCR, real-time PCR is not able to discriminate between culturable versus VBNC and dead cells, as well as naked DNA (that may amplify if the nucleic acid is not completely degraded) which can affect estimation in a quantitative assay. Furthermore, the presence of substances that inhibit PCR, often present in environmental samples, can also result in a false negative (Lyon, 2001). Few studies have been designed and validated for a quantitative real-time PCR method for detection and quantification of *V. cholerae*. Oligonucleotide primers and probes for Real-Time PCR assays are listed in Table 4.

We highly recommend conducting all real-time PCR tests in a UV hood as well as sterilizing all equipment (making sure to not expose reagents, primers, or probes to ultraviolet radiation) used for real-time PCR. This reduces risk of contamination.

BASIC PROTOCOL 6: TaqMan assay for detection of *V. cholerae* (Lyon 2001)

The TaqMan assay is a real-time PCR method based on 5' exonuclease activity of *Thermus aquaticus* DNA polymerase used to hydrolyze an internal probe labeled with a fluorescent reporter dye (FAM, Cy3, Cy5, TET) and a quencher dye. During PCR amplification, hydrolyzation of the probe separates the reporter dye from the quencher dye which results in increasing fluorescence proportional to the amount of template DNA present in the reaction, thereby giving a quantitative estimation. Lyon et al. (Lyon 2001) developed a method for a quantitative, sensitive, and rapid detection of *V. cholerae* in pure cultures, seawater, raw oysters with primers and probes directed toward the non-Classical specific hemolysin (*hylA*) gene of *V. cholerae* O1, O139 and non-O1/O139 (Table 4). Another quantitative TaqMan assay was developed for detection of the *ctxA* gene in seawater and oysters with the sensitivity of <10 CFU per reaction (Blackstone, Nordstrum et al. 2007).

Materials

TaqMan® PCR Reagent Kit

10X TaqMan buffer A

25 mM $MgCl_2$

dATP, dCTP, dGTP, and dUTPs

Taq DNA polymerase

AmpErase UNG (1U/μL)

Forward Primer

Reverse Primer

probe (FAM)

The TaqMan *V. cholerae*-specific probe is an oligonucleotide with a 5' reporter dye (FAM-6-carbooxyfluorescein) and a 3' quencher dye (TAMRA-6-carboxy-*N'N'N'N'*-tetramethylrhodamine).

Optical reaction tubes or plates

Optical reaction tube caps or plate seal

Real-Time PCR (qPCR) machine

1. Set up Real Time PCR with a total amplification reaction mixture of 50-μl per sample containing the following.2.5μl DNA sample (100 ng/μl) [Copy Editor: Please ask author if you use the crude prep in SP1 or the DNA as prepared in BP 8.]

1X TaqMan buffer A

5 mM $MgCl_2$

200 μM (each) dATP, dCTP, and dGTP; 400 μM dUTP

0.02 μM *hylA*-probe

0.3 μM *hylA*-F primer

0.3 μM *hylA*-R primer

1 U of AmpErase uracil N-glycosylase

2.5 U of AmpliTaqGold DNA polymerase

2. Amplify the targets with the following cycle conditions:

Initial hold:	5 min at 50°C
Initial denaturation:	5 min at 94°C
40 cycles:	20 sec at 95°C
	1 min at 60°C

3. Results are interpreted using computer software available with Real-Time PCR machines by monitoring increase in fluorescence throughout the amplification cycles and reported as C_t value. The C_t value is the number of amplification cycles required to detect a fluorescent signal above a given threshold. C_t levels are inversely proportional to the amount of target nucleic acid in the sample. In general, C_t values < 29 are considered strong positive reactions and are indicative of abundant target nucleic acid in the sample, while C_t values of 30 to 35 are positive reactions indicative of moderate amounts of the target, and C_t

values of 38 to 40 are considered weak reactions with little or no target nucleic acid in the sample (Figure 5).

BASIC PROTOCOL 7: SYBR Green assay for detection of *V. cholerae* (Gubala, 2006)

An alternative Real-Time PCR method involves the use of a dsDNA binding dye such as SYBR Green I. In this technique the amplification products are distinguishable by analysis of their melting temperature (Gubala, 2006). This assay was used in two studies to develop a multiplex real-time PCR of four and six target genes for *V. cholerae* and other vibrios detection in sea, estuarine and river water samples, and seafood (Table 4) (Gubala and Proll 2006; Gubala 2006).

Materials

LightCycler® FastStart DNA Master SYBR Green I

LightCycler® FastStart Enzyme

LightCycler® FastStart Reaction Mix SYBR Green

$MgCl_2$ Stock Solution, 25 mM

H_2O, PCR-gradeTaq DNA polymerase

Forward Primer

Reverse Primer

Optical reaction tubes or plates

Optical reaction tube caps or plate seal

Smart Cycler (Cepheid, Sunnyvale, Calif.)

1a. For a single target, prepare the amplification reaction mixtures: 25 µl containing:

1 µl template DNA.

0.2 µM of each primers,

2.5 µl of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Laval, Quebec, Canada),

3.75 mM $MgCl_2$ (final concentration)

1b. For Multiplex PCR, prepare Amplification reaction mixtures: 25 µl containing:

1 µl template DNA.

0.08 µM each *rtxA* primers;

0.15 µM each *epsM* primers;

0.40 µM each *mshA* primers;

0.20 µM each *tcpA* primers;

3 µl of LightCycler FastStart DNA Master SYBR Green I,

4.0 mM MgCl2 (final concentration).

2. Amplify the targets with the following cycle conditions:

initial denaturation: 150 sec at 95°C	
45 cycles (single assays)	15 sec at 95°C

35 cycles (multiplex assay):	30 sec at 60°C
	30 sec at 72 °C

3. Results are interpreted using designated computer software. As with the TaqMan assay, results are expressed in C_t values, the number of amplification cycles required to detect a fluorescent signal above a given threshold. In a multiplex SYBR Green real-time PCR, the amplification products are distinguished based on their melting temperature (T_m). The T_m values of each of the products are calculated at the completion of PCR amplification monitoring the fluorescence in increasing temperature between 60°C and 95°C, at a given rate. The T_m peaks are calculated based on the initial fluorescence curve.

BASIC PROTOCOL 8: Direct PCR for Environmental Samples

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 *wbe*); and toxin and pathogenic factor genes (*ctx, tcpA*, etc.). Briefly, water, plankton, oyster, and/or sediment samples are collected and concentrated. DNA is extracted from the samples, using a modification of the method of Murray and Thompson (Murray and Thompson 1980) and PCR is performed on the extracted DNA using a multiplex (*ompW* and *ctxA*) primer array. The PCR protocols described in Basic Protocol 5 (Sub-Protocols 1 and 2 and Alternative Sub-Protocol 1) are used and the DNA template is derived from the DNA extraction method described in this Basic Protocol (below). 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 3 for PCR primers and reference)

Materials

Pre-sterilized glass (240-ml, Qorpak, Bridgeville, PA) and plastic (500-ml, Nalgene, Rochester, NY) containers

Simple Plankton net, 64 μm, or nets of different mesh sizes (for size filtration of plankton) (Aquatic Research Instruments, Florida, USA) (See Figure 2)

Portable meter(s) to measure temperature, dissolved oxygen, pH, and salinity (HACH Company, Colorado, USA)

Bucket of known volume (5 liter) (optional)

Tissue homogenizer (hand held or powered) to homogenize plankton (Kontes, Vineland, NJ)

Filter apparatus with vacuum source

Filter membranes, 47-mm diameter, 0.22-μm pore size

Sterile 150-ml Erlenmeyer flasks containing 25-ml APW

Alkaline peptone water (APW), pH 8.6

TE buffer (*APPENDIX 2A*)

10% SDS

Proteinase K solution (20-mg/ml, 2%)

5 M NaCl

CTAB/NaCl solution

Chloroform/isoamyl alcohol (24:1)

Phenol/chloroform/isoamyl alcohol (25:24:1)

Isopropanol

70% ethanol

20 μM PCR primers (Table 3)

25 mM dNTPs (*APPENDIX 2A*)

Taq DNA polymerase

10× Reaction Buffer, 500-mM KCl, 100-mM Tris-HCl pH 8.3 (at 25°C), 15-mM Mg^{2+}

Sterile water

Molecular weight ladder (Hyperladder IV, Bioline, Taunton, MA)

Thermal cycler (BioRad, Hercules, CA)

Microcentrifuge tubes

Boiling water bath

PCR tubes

Horizontal gel apparatus, gel tray and comb.

Power supply for gel apparatus

1 μM ethidium bromide staining solution (*APPENDIX 2A*)

UV transilluminator (UVP, Upland, CA)

Collection and concentration of environmental sample—*For water and plankton samples*

1a. Collect sample as described in the conventional bacterial culture method above (Basic Protocol 1).

2a. Perform enrichment in APW using the conventional bacterial culture method (Basic Protocol 1).

For sediment samples

1b. Add sediment to 100-ml of distilled water until the final volume reaches 200-ml. Mix well and allow the sediment to settle. Remove particulate matter by centrifuging a 10-ml aliquot of the slurry for 8 min at 1000 × *g*, room temperature.

2b. For sediment samples, enrich 1-ml of the sediment slurry in an APW enrichment flask, as for water and plankton samples.

For oyster samples

1a. Collect sample as described in the conventional bacterial culture method above (Basic Protocol 1)

2b. Perform enrichment in APW using the conventional bacterial culture method (Basic Protocol 1)

Processing these samples in 10-fold dilutions and in triplicate or quintuplicate (i.e., triplicates or quintuplicates of 100, 10, and 1 ml of water) followed by direct PCR on these dilutions will allow a quantitative estimation of the Most Probable Number (MPN) PCR target in each sample. This can be done for all collected sample types and dilutions for

oysters and sediments would be 10-fold weight increments (i.e., 10, 1, 0.1 g of homogenized oysters. For more information on the Most Probable Number assay see Standard Methods for the Examination of Water and Wastewater (Clesceri and Greenberg, et al. 1998).

DNA extraction using phenol, chloroform, and isoamyl alcohol

3. Microcentrifuge a 1-ml aliquot from the upper surface (i.e., top 1–2 mm) of the APW enrichment for 5 min at 12,000 × *g*, room temperature.
4. Resuspend the cell pellet in 567 µl TE buffer.
5. Add 30 µl of 10% SDS, followed by 3 µl Proteinase K solution.
6. Incubate the suspension 1 hr at 35°C.
7. Add 100 µl of 5M NaCl, followed by 80 µl of CTAB/NaCl solution.
8. Incubate mixture 10 min at 65°C.
9. Add 800-µl phenol/chloroform/isoamyl alcohol (25:24:1), vortex and centrifuge 5 min at 12,000 × *g*, room temperature.
10. Transfer aqueous phase (supernatant) 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.
11. Transfer aqueous phase (supernatant) to a new microcentrifuge tube. Precipitate DNA with an equal volume of isopropanol.
12. Pellet DNA by centrifuging 5 min at 12,000 × *g*, 25°C. Wash DNA with 1 ml of 70% ethanol and centrifuge 5 min at 12,000 × *g*, 25°C.
13. Dry pellet in vacuum desiccator or lyophilizer and resuspend in 100 µl TE buffer.

Quick DNA extraction

1. Remove a 1-ml aliquot from the upper surface (i.e., top 1–2 mm) of the APW enrichment.
2. Boil for 10 minutes.
3. Put on ice, then centrifuge to pellet cell material.
4. Remove ca. 700-µl supernatant and add this to a sterile 1.5-ml microcentrifuge tube. *This supernatant contains community DNA.*
5. Add 28-µl of 10-mg/mL bovine serum albumin (BSA) (4-µl per 100-µl supernatant). *It is also beneficial to make a 1:10 dilution (supernatant:sterile DI water) and use this for PCR as well as a dilution may help to remove PCR inhibitors that can be present in community DNA samples.*

Perform Direct PCR

1. Perform the PCR protocols described in Basic Protocol 4 or Alternate Protocol 2 or Basic Protocol 5 on the extracted DNA template. Additionally, PCR assays described in Supplementary Table 1 can be explored to evaluate the presence of other *V. cholerae* virulence genes not targeted in these three protocols.

BASIC PROTOCOL 9: 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 culture based, so it is dependent upon the presence of *V. cholerae* in the sample as viable, culturable cells. Its 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 media should be relatively rich and non-selective to allow for vigorous growth and cells with a high RNA content. Nitrocellulose (or nylon) membranes are overlaid, lifted, and treated to bind RNA (Rehnstam, Norqvist et al. 1989) (or DNA) to the membrane. Plates to be lifted should contain 50 to 150 well-defined colonies, 2.0–3.0-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, Heidelberg et al. 2002) or toxigenic *V. cholerae* (*ctxA*). *V. mimicus* is a closely related species to *V. cholerae* which was previously described as biochemically atypical *V. cholerae* (non-sucrose 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 CPMB 3.18); however, the DIG system (Roche) offers a good alternative when a variable mode imager (such as Typhoon, GE Healthcare) or an alternative machine, such as Dark Reader (Clare Chemical Research, Colorado, USA), for detection of the fluorochrome is not available. The *V. cholerae*-specific RNA colony blot/hybridization protocol is presented first and then a *ctxA*-DNA colony blot/DIG (Roche) hybridization protocol is presented.

Materials

85-mm sterile nitrocellulose membranes, 0.22-μm (GE Osmonics)

LB or modified nutrient agar plates

10% SDS

3X SSC (*APPENDIX 2A*)

65°C incubator

70°C oven

Pre-washing solution

Hybridization solution base

DEPC-treated water (*APPENDIX 2A*)

Washing solution

Fluorescein-labeled Vchomim1276 probe, reconstituted at 25-ng/μl

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.

Spread-plate preparation

1. For detection, prepare spread plates from APW enrichment flasks using APW as diluent and plating three serial-fold dilutions onto LB or modified nutrient agar plates.
2. For enumeration (and detection), spread plate appropriate dilutions onto LB or modified nutrient agar plates on-site without APW enrichment.

   Alternatively, 100–500-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 membrane and plate overnight at 30°C and then proceed to Step 7.
3. Incubate plates overnight at 30°C

Colony lift

4. Mark membranes using a lead pencil with Blot ID (e.g., medium, sample, dilution) that matches plate to be lifted and orientation marks (asymmetrical).
5. Overlay membrane, starting from the center to ensure there are no air bubbles.
6. Allow at least 15 minutes for transfer.
7. Replica-plate the membrane onto a fresh modified nutrient agar plate, transferring orientation markings.
8. Preheat SDS, SSC, and Pyrex dishes (for holding filter paper) to 65°C.
9. Place membrane, colony side up, on the filter paper (cut to just slightly larger than the membrane) pre-wetted with 10% SDS, enclosed in a Pyrex dish, for 5 minutes at 65°C. Cover Pyrex dish containing wetted filter paper and membrane with Saran wrap or equivalent film to prevent the filter paper from drying out.

   It is important to use filter paper pre-wetted, but not saturated with 10% SDS or 3X SSC (next step) to prevent colonies from over-swelling and losing their circular shape. Pour or pipet the liquid onto the filter paper, let soak briefly, remove air bubbles, and then pour off excess. Ensure that there is no pooled liquid on the filter paper prior to placing membrane.
10. Incubate the membrane 15 min at 65°C on fresh filter paper wetted with 3X SSC. Cover Pyrex dish containing wetted filter paper and membrane with Saran wrap or equivalent film to prevent the filter paper from drying out.
11. Let membranes air-dry 10 min on filter paper.
12. Bake membranes 15 min at 70°C.

RNA-Colony Blot Hybridization

13. Wash membranes three times in adequate pre-washing solution for 15 minutes at room temperature.
14. Wash membranes 1.5 hr at 60°C in prewashing solution.
15. After pre-washing steps, rinse membranes in DEPC-treated water.

    Hybridization solution base will form a precipitation upon maintaining at room temperature. If this happens, heat to 40–50°C to resuspend.
16. Pre-hybridize the membranes 30 min at 60°C in hybridization solution at a ratio of 10-ml pre-hybridization solution per 100-cm^2 of blot membrane.

85 mm-membranes have a surface area of ~60-cm^2.

17. Pour off pre-hybridization solution and add hybridization solution plus probe (32-µl/10-ml solution) at a ratio of 10-ml hybridization solution per 100-cm^2 blot.

18. Hybridize overnight (16–20 hours ideally) at 60°C.

19. After hybridization, wash membranes 30 min at 60°C in washing solution.

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.

ALTERNATE PROTOCOL 3: Colony Blot Hybridization with using DIG-labeled *ctx* DNA probe

The previous colony blot hybridization protocol (Basic Protocol 9) 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 following the method of Pal et al, 1992 (Pal, Ramamurthy et al. 1992). The hybridization is done according to the DIG protocol (Roche). The protocol is given here; readers are encouraged to consult the manual accompanying the High Prime kit. The DIG DNA probe-labeling protocol is given in a support protocol. The *ctxA* probe can be produced from PCR, using the pCTA primer set (see Table 3) or from *Eco*RI digestion of plasmid, pKTN901, which contains a 540-bp *Xba*I-*Cla*I fragment of *ctxA* (Kaper, Morris et al. 1988).

Materials

Luria-Bertani (LB) agar plates (*APPENDIX 4A*)

Sterile nylon (or nitrocellulose) membranes, 85mm, 0.22-µm (GE Osmonics)

Whatman filter paper, #3

Lysis buffer

Neutralization solution

UV crosslinker or transilluminator

1X SSC buffer (*APPENDIX 2A*)

Proteinase K solution

Incubators, 37°C and 42°C

Shaking water bath

DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, kit includes DIG Easy Hyb granules, 10X Blocking solution, CSPD, anti-digoxigen-AP conjugate, DIG High Prime)

Stringency wash solution I

Stringency wash solution II

Maleic Acid Buffer

Washing Buffer

Blocking Solution, 1X in Maleic Acid Buffer

Antibody Solution, 1:10000 anti-digoxigenin-AP conjugate in Blocking Solution

Detection Buffer

X-ray film (Kodak or Fuji)

Film developer

Blot Preparation

1. Prepare spread plates as above, using LB agar plates (See Basic Protocol 9, steps 1 to 3).
2. Incubate the plates overnight at 37°C
3. Mark membranes using a lead pencil, including Blot ID (e.g., medium, sample, dilution) that matches plate to be lifted and orientation marks (asymmetrical).
4. Overlay membrane, starting from the center to ensure there are no air bubbles.
5. Transfer at least 15 min.
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 10–15°C.

   Master plates can be kept like this for up to 2 weeks.
7. Place membranes, colony side up, onto #3 Whatman filter paper pre-soaked with lysis buffer. Incubate 10 min at room temperature.
8. Remove the membrane from lysis buffer and place onto #3 Whatman filter paper presoaked with neutralization solution
9. Repeat step 8.
10. Remove membrane from neutralization solution and place onto #3 Whatman filter paper and air dry (approximately 30 minutes).
11. Immobilize colonies onto the membrane using a UV crosslinker or transilluminator.

    Damp membranes should be crosslinked at an output intensity of 120-mJ/cm2, which corresponds to the optimal or auto-crosslink setting on commercially calibrated 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 two times in 1X SSC buffer and air dry (approximately 30 minutes).
13. Treat membranes 30 min at 42°C with 100-ml Proteinase-K solution using gentle shaking.
14. Rinse filters three times in 1X SSC in a shaking water bath at room temperature for 10 min each. Let air dry (approximately 30 minutes).

Prehybridization and Hybridization

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 probe by boiling for 5 min and rapidly cooling on ice.
18. Pour off pre-hybridization solution.
19. Add fresh DIG-Easy Hybridization Solution plus denatured probe (25 ng/ml solution) and incubate overnight at 42°C.

Stringency washes

20. Pour off probe-containing hybridization solution and store up to 2 months at −20°C.

    Probe-containing hybridization solution can be reused several times.
21. Wash membrane two times in Stringency Wash Solution I for 5 min at room temperature under constant agitation.
22. Wash two times in Stringency Wash Solution II for 15 min at 65°C under constant agitation.

Detection

23. Rinse membrane briefly (5 min) in Washing Buffer.
24. Incubate the membrane 30 min at 25°C in Blocking Solution.
25. Incubate the membrane 30 min at 25°C in Antibody Solution for 30 min.
26. Wash the membrane two times in Washing Buffer, for 15 min each.
27. Equilibrate the membrane in Detection Buffer for 5 min at 25°C.
28. Place membrane in hybridization pouch. Add CSPD 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 over-exposed, repeat step 30 and vary time of exposure accordingly.
31. From master plates (step 6), subculture colonies that were positive by blot hybridization for further analysis.

SUPOORT PROTOCOL 2: DNA-labeling of ctxA probe using DIG-High Prime

PCR or digestion-generated probes can be labeled by DIG-High Prime (Roche), 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. Alternately, 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, Morris et al. 1988), which has been widely used (Pal, Ramamurthy et al. 1992; Islam, Rahman et al. 2005). The *Xba*I-*Cla*I fragment can be removed from the plasmid by digestion with *Eco*RI and gel purified. Labeled probes can then be used in the DIG-based hybridization above.

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

Sterile distilled water

Boiling water bath

DIG-High Prime

Ice/water bath

Incubator, 37°C

0.2 M EDTA, pH 8.0

1. For PCR-generated *ctxA* target fragment, amplify extracted DNA of a toxigenic (*ctxA*$^+$) *V. cholerae* reference strain using the pCTA primer set.
2. For digestion-generated *ctxA* target fragment, digest plasmid, pKTN901, with *Eco*RI for 2 hrs at 37°C
3. Clean-up PCR reaction product or digestion product by gel electrophoresis followed by gel extraction (CPMB 2.6).
4. Bring 1-μg of purified *ctxA* fragment up to a total volume of 16-μl in sterile distilled water.
5. Denature by placing in a boiling water bath for 10 min, followed by rapidly chilling in an ice bath (5 min).
6. Add 4-μl of DIG-High Prime to the DNA solution, mix, and centrifuge briefly to collect liquid.
7. Incubate overnight at 37°C.
8. Stop reaction by adding 2-μl of EDTA and/or by heating to 65°C for 10 min.

IMMUNOLOGICAL METHODS FOR DIRECT DETECTION OF *V. CHOLERAE* IN ENVIRONMENTAL SAMPLES

Conventional culture methods are ineffective when bacterial cells have entered into the viable but non-culturable (VBNC) state. Thus, direct detection becomes extremely important. Despite the ubiquitous nature of *V. cholerae*, isolation and detection by traditional methods are difficult since these methods rely on culturing the organism. The discovery of monoclonal antibody in the 1980s and, subsequently, the development of a monoclonal antibody against *V. cholerae* O1 triggered development of direct detection methods for this bacterial species (Xu, Roberts et al. 1984; Hasan, Loomis et al. 1992). Using immunological methods, the mystery concerning the inability to culture *V. cholerae* in environmental samples was reported during inter-epidemic period in Bangladesh (Roszak and Colwell 1987; Huq, Colwell et al. 1990). These difficulties arise from several possible factors: low density, inter-specific competition, cell state, and health (VBNC, starved). Fluorescent In-Situ Hybridization (FISH) allows direct detection of taxa-specific nucleic acid followed by visualization using microscopy and the polymerase chain reaction offers a molecular-based alternative to the traditional culture and immunological methods (discussed later).

BASIC PROTOCOL 10:Fluorescent In-Situ Hybridization (FISH) Detection of V. cholerae

Direct quantification methods without the need to enrich and culture can yield highly accurate and quick estimates of taxon-specific densities in water bodies. Fluorescence *In-Situ* Hybridization (FISH) accomplishes this with a fluorescently-labeled oligonucleotide probe that is visualized under epifluorescence or confocal laser scanning microscopy. This method is reliable for accurate estimations of *V. cholerae* cells in media as it allows one to quantify both culturable and non-culturable (VBNC) *V. cholerae* cells.

Materials

4% paraformaldehyde solution in 1X PBS

1X PBS

1X PBS-ethanol solution (1:1, PBS: absolute ethanol)

1.5 or 2-ml microcentrifuge tubes

FITC-labeled probe, Vchomim1276, 6-FAM (fluorescein phosphoramidite) labelled probe (5'-[5FITC] ACTTTGTGAGATTCGCTCCACCTCG-3') at a working concentration of 50 ng/μl in sterile water (final concentration, 5 ng/μl).

Multiwell slides

0.01% poly-L-lysine solution (PLL)

Ethanol solutions (50, 80, and 96%)

Hybridization solution

Washing buffer solution

Anti-fade agent, Citifluor AF1

50-ml centrifuge tubes

Whatman filter paper

Incubator or water bath set to 45°C.

Sample preparation

1. Filter water (ca. 500 to 1000 ml) through a 0.2 μm polycarbonate membrane, and resuspend cells attached to membrane in 5 ml 1X PBS.
2. Centrifuge 1 ml of 1X PBS re-suspension at 13,000 g for 10 min in microcentrifuge tube. Discard supernatant and resuspend in 250 μl of 1X PBS
3. Fix cells by adding 750 μl of fresh 4% paraformaldehyde solution and incubate at room temperature (22–25°C) for 1hr.
4. Next, pellet the cell solution by centrifugation at 13,000 g for 5 min. Discard the supernatant and then wash the cell twice with 1X PBS.
5. Concentrate the cells by centrifugation and re-suspend in 100 μl PBS-ethanol solution.
6. Store cells at −20°.

Hybridization

1. Immerse multiwell slides in 0.01% PLL solution for 10 min and air-dry.
2. Spot 5 μl aliquots of fixed cells onto slides and air-dry.

3. Wash slides for 3 min each in successive ethanol solutions (50, 80 and 96%) and then air-dry. Pre-warm humid chamber, slides and filter paper soaked with hybridization solution at 45°C for 10 minutes.
4. To each well, add 5 µl aliquot of Hybridization solution containing 5 ng/µl of labeled probe.
5. Perform hybridization at 45°C for 24 hrs in the humid chamber using hybridization soaked paper.
6. Pre-warm the washing buffer solution at 45°C for 10 minutes before washing the slides and incubate at 45°C for 10 minutes.
7. Wash slides in sterile deionized water and air-dry at room temperature.
8. Add anti-fading agent to each well and a cover slip is applied.
9. Fluorescence is visualized under epifluorescence microscopy (Fig. 6.)

BASIC PROTOCOL 11: 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 non-culturable cells (VBNC) of *V. cholerae* (Chowdhury, Hasan et al. 1994). It is a two-step method where samples are incubated with yeast extract in the presence of nalidixic acid, after which actively viable, substrate-responsive cells become enlarged and elongated (Kogure, Simidu et al. 1979). Next, an aliquot from this suspension are air dried on a glass slide and stained with fluorescent labeled monoclonal antibody, raised against 'A' factor of *V. cholerae* O1 lipopolysaccharide that reacts with both serotypes, Ogawa and Inaba (Colwell, Tamplin et al. 1990; Hasan, Bernstein et al. 1994). Antibodies against *V. cholerae* O139 are also available (Hasan, Huq 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. 7). *V. cholerae* O1 and O139 DVC-DFA positive samples can be confirmed by PCR (Binsztein, Costagliola 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 (CholeraDFA) and O139 (BengalDFA) DFA tests are commercially available (New Horizon Diagnostics, Columbia, MD).

Materials

Concentrated water or homogenized plankton sample (Basic Protocol 2)

Yeast extract, 2.5% solution in distilled water

Nalidixic acid, 0.2% solution in distilled water

35°C incubator

Formaldehyde solution, 37–40%, or fresh 4% formaldehyde solution made from paraformaldehyde

Micropipettes

Multiwell slides and cover slips

Absolute methanol

Distilled or deionized water

Phosphate buffered saline (PBS; *APPENDIX 2A*)

Humid chamber: 50-ml centrifuge tube containing 1–2 strips of saturated filter paper

CholeraDFA and/or BengalDFA kit (test kit comes with FITC-conjugated DFA reagent, positive and negative control, slides and fluorescent mounting medium; New Horizon Diagnostics).

Epifluorescent microscope, with FITC filter set

NOTE: All solutions should be 0.1 μm filtered and sterile, 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 1), add 10-μl yeast extract solution and 10-μl nalidixic acid solution.
2. Freeze (-20°C) a parallel sample (1-ml) for PCR confirmation.
3. Incubate the mixture at 25°C for a minimum of 6 hrs to overnight.
4. 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

5. Place 5 to 10 μl of the fixed sample onto a glass slide and air dry (approximately 15–20 minutes).
6. Fix by adding 5 μl methanol and air dry (1–5 minutes).
7. Add 10-μl of reconstituted FITC-conjugated specific DFA reagent (Hasan, Bernstein et al. 1994), as supplied by Cholera DFA or BengalDFA kit.
8. Incubate 30 min at 37°C in humid chamber. Protect slide from light.
9. Rinse slide with ~50 ml PBS (each). Air dry slides in the dark (approximately 15–20 minutes).
10. Mount slide with one drop of kit-provided Fluorescent Mounting Medium and add cover slip.
11. Observe under an epifluorescent microscope (see Fig 7).

BASIC PROTOCOL 12: Indirect Fluorescent Antibody (IFA) Method

This immunofluorecent method for detection of *V. cholerae* serogroup O1 in aquatic environmental samples was first introduced by Xu *et al.* in 1984 (Xu, Roberts et al. 1984). Antiserum specific for O1 somatic antigen produced in rabbits was used with fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit globulin goat serum, with rhodamine isothiocyante (RITC)-conjugated bovine serum albumin as the background stain. This method was found to be very useful for detecting organisms in samples which gave negative results by culture (Brayton, Roszak et al. 1986; Huq, Colwell et al. 1990). It was later optimized and packaged as the direct fluorescent antibody staining kit for *V. cholerae* O1, Cholera DFA by Hasan et al. (Hasan, Bernstein et al. 1994). 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)

Multiwell, Teflon-coated slides

Glass coverslips

Phosphate buffered saline (PBS; *APPENDIX 2A*)

95% ethanol

Incubators, set to 55°C and 35°C

50-ml screw-cap centrifuge tubes

Filter paper, cut into thin strips to fit inside centrifuge tubes

FA Rhodamine counterstain (Becton Dickinson)

Polyvalent *V. cholerae* O1 antiserum (Difco)

FITC-conjugated anti-rabbit globulin goat serum (Sigma)

Mounting medium, such as FA (Difco) or Citifluor AF1/AF3 (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA)

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 (15–20 minutes) at room temperature.
2. Fix the sample by adding 95% ethanol to each well containing sample. Air dry (5–10 minutes) 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.

Staining procedure

4. Rinse the slide(s) with ~50-ml PBS and air dry (15–20 minutes).
5. While slide is drying, prepare humid chamber for use. Equilibrate 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 Counterstain. Incubate in humid chamber 30 min at 35°C.

   Minimize exposure to light from this step forward.
7. Rinse slide in PBS by gently flooding (~50-ml), then soak in the same solution 10 min at room temperature. Remove and rinse again briefly in PBS.
8. Allow slide to air dry (15–20 minutes).
9. Add 5–10-μl of *V. cholerae* O1-specific antiserum. Incubate in humid chamber 30 min at 35°C.
10. Repeat steps 7 and 8. Use fresh PBS for washing.
11. Add 1–2 drops undiluted FITC-conjugated anti-rabbit globulin goat serum and incubate in humid chamber 30 min at 35°C.

12. Repeat washing steps 7 and 8 using fresh PBS.
13. Mount each slide with a glass cover slip 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.

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 (1×)

Peptone, 10-g

Sodium chloride, 10-g

Adjust volume to 1 liter with water

Adjust pH to 8.6 with NaOH

Autoclave to sterilize

Store up to 6 months at 2–8°C

Alkaline Peptone Water (10×)

Peptone, 100-g

Sodium chloride, 100-g

Adjust volume to 1 liter with water

Adjust pH to 8.6 with NaOH

Autoclave to sterilize

Store up to 6 months at 2–8°C

Estuarine Peptone Water (EPW)

autoclaved natural estuarine water amended with 1% peptone

Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar

Yeast extract, 5-g

Peptone, 10-g

Sodium thiosulfate, 10-g

Sodium citrate, 10-g

Ox Bile, 8-g

Sucrose, 20-g

Sodium chloride, 10-g

Ferric citrate, 1-g

Bromothymol blue, 0.04g

Thymol blue, 0.04-g

Agar, 14-g

Adjust volume to 1 liter with water

Boil to completely dissolve

IMPORTNANT NOTE: Do not autoclave.

This medium is also available commercially as a dehydrated powder from Oxoid (Remel, Lenexa, KS, USA)

Modified Nutrient Agar

Beef extract, 3-g

Peptone, 5-g

Sodium chloride, 10-g

Agar, 15-g

Adjust volume to 1 liter with water

Autoclave to sterilize

Store up to 1 month at 2–8 °C

Monsur's Tellurite Taurocholate Gelatin Agar (TTGA)

Tryptone, 10-g

Sodium chloride, 10-g

Sodium taurocholate, 5-g

Sodium carbonate, 1-g

Gelatin, 30-g

Agar, 15-g

Adjust volume to 1 liter with water

Boil to completely dissolve ingredients

Final pH should be 8.5. If it is not, adjust with HCl

Autoclave and add potassium tellurite to 1% (w/v) final

Store up to 1 month at 2–8 °C

Gelatin Agar (GA)

Neopeptone, 4-g

Yeast extract, 1-g

Sodium chloride, 5-g

Gelatin, 15-g

Agar, 15-g

Adjust volume to 1 liter with water

Autoclave to sterilize

Store up to 1 month at 2–8 °C

Modified HPCA medium

Peptone, 3.0-g

Soluble casein, 0.5-g

Potassium phosphate dibasic, 0.2-g

Magnesium sulfate, 0.05-g

Ferric chloride, 0.001-g

Sodium chloride, 10-g

Agar, 15.0-g

Adjust pH to 7.2 with HCl

Autoclave to sterilize

Store up to 1 month at 2–8 °C

Hybridization solution

0.9M NaCl

20 mM Tris-HCl

0.01% SDS, pH 7.2,

35% Formamide

Washing buffer solution

0.9M NaCl

20mM Tris-HCl

0.01% SDS

1X PBS-ethanol solution

50% 1X PBS

50% absolute ethanol

Methyl Red indicator solution

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 2–8°C.

Pre-washing solution

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

SSC, 3X (*APPENDIX 2A*), RNase-free

SDS, 0.1%, RNase-free

Prepare fresh

Hybridization solution base

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

NaCl, 0.9-M

Sodium phosphate (pH 8.0), 50-mM

EDTA, 5-mM

SDS, 0.5%

Store up to 1 month at room temperature

Precipitation will occur upon standing at room temperature. Heat to 45° to 50°C to resuspend.

Washing solution

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

SSC, 1X (*APPENDIX 2A*)

SDS, 0.1%

Prepare fresh

CTAB/NaCl Solution

Add 4 g NaCl to 80-ml water and dissolve. Slowly add 10 g of 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.

Fluorescein-labeled Vchomim1276 probe

5’-ACTTTGTGAGATTCGCTCCACCTCG-3’, with 5’ Fluorescein label.

Resuspend probe to a working concentration of 25-ng/μl. Add probe to hybridization solution base at a ratio of 32-μl per 10-ml of solution.

Cell Lysis Buffer

Sodium hydroxide, 0.5-N

Sodium chloride, 1.5-M

Prepare fresh

Blot Neutralization Solution

0.5 M Tris-HCl, pH 7.2 (*APPENDIX 2A*)

Sodium chloride, 1.5-M

Store up to 6 months at 2–8°C.

Proteinase K Solution (40-μg/ml)

Proteinase K, 4-mg

1× SSC (*APPENDIX 2A*), 100-ml

Store up to 2 months at −20°C.

Stringency Wash Solution I

SSC, 2X (*APPENDIX 2A*)

SDS, 0.1%

Prepare fresh

Stringency Wash Solution II

SSC, 0.5X (*APPENDIX 2A*)

SDS, 0.1% (w/v)

Prepare fresh

Washing Buffer

Maleic Acid, 0.1-M

Sodium chloride, 0.15-M

Tween 20, 0.3% (v/v)

Adjust pH to 7.5 with solid NaOH

Prepare fresh

Maleic Acid Buffer

Maleic Acid, 0.1-M

Sodium chloride, 0.15-M

Adjust pH to 7.5 with solid NaOH

Prepare fresh

Detection Buffer

0.1 M Tris-HCl, pH 9.5 (*APPENDIX 2A*)

Sodium chloride, 0.1-M

Store up to 1 month at room temperature

COMMENTARY

Background Information

Understanding the natural ecology of an infectious agent outside of the human body is essential to understanding the epidemiology of the associated disease and especially necessary in any attempt to prevent illness caused by exposure to that pathogen. It is well established that *V. cholerae* is autochthonous to the aquatic environment globally and even in regions where cholera is absent (Haley, Chen et al. 2012). However, these environments are highly heterogeneous and change over time. It is also well established that seasonal fluctuation in environmental parameters is associated with changes in environmental pathogen densities and disease prevalence. It is, therefore, necessary to understand the influence of the environment on presence and number of these organisms. This requires an holistic approach toward investigation of the microbial ecology of an infectious disease. Temporal and spatial studies of *V. cholerae* in water, planktonic organisms, sediment, and shellfish, coupled with recording physical and chemical parameters of the study environment (regardless of presence or absence of *V. cholerae*) allows investigators to estimate changes in the presence or density of *V. cholerae* in a particular region. This information can be used to help predict the public health safety of water bodies or seafood, thereby reducing risk of illness and loss of revenue from inaccurate prediction of the presence of pathogens in water bodies used for recreation or shellfish harvesting. Although, cholera is a public health threat for developing nations more than for developed nations, methods for *V. cholerae* discussed in this chapter can be applied to other waterborne pathogens with addition of methods specific to the microorganism of interest.

As commented above, multiple approaches should be taken to study the ecology of pathogens in the environment. The coupling of methods can overcome limitations inherent in any one particular method and allow convergence on the precise answer(s). However, increase in the cost and time commitment needs to be considered. An investigator should first determine the data or results needed, appropriateness of available methods, and feasibility of carrying out the procedures to achieve the goal. If the microorganism of interest is not detected, it is not appropriate to conclude that the pathogen is absent from study, but rather that it was not detected by the methods used. In this scenario, use of multiple methods reduces "not detected" results. Further, parallel replication of assays will enhance accuracy and statistical power of the results.

Critical Parameters and Troubleshooting—Successful detection and isolation of *V. cholerae* is dependent on both its presence in the environment as well as the method used for detection. *V. cholerae* undergoes the VBNC state at prolonged low temperatures, which will influence whether culture of the bacterium will succeed when water temperatures are low. Lack of growth on media is indicative of *V. cholerae* in the VBNC state rather than an absence of *V. cholerae* in the water body of interest. In such instances, inclusion of direct detection methods (DFA, IFA, FISH, direct PCR) will provide more precise results with respect to the presence of *V. cholerae*. If such methods are used in conjunction with culturing methods then the methods should be used throughout the entirety of the study rather than alternating methods based on the prevailing environmental conditions.

The culturing methods presented in this unit are the most commonly used culture-based protocols for isolation of *V. cholerae* (Morris, Merson et al. 1979; Rennels, Levine et al. 1980). Other methods have been described and may be suitable for certain study environments. Alkaline bile peptone water (Spira, Huq et al. 1981), Monsur's tellurite taurocholate broth (Monsur 1961), and sodium-gelatin phosphate broth (Rennels, Levine et al. 1980) have been described as enrichment media that are effective for culturing *V. cholerae*. A second enrichment step may be used, but will add an additional 6 to 8 hr to an already lengthy protocol. There is also the concern that repeated passaging of cells will allow for population and genotypic alterations to occur by favoring cells which grow more readily in enrichment broths, allowing for mutations to accumulate, i.e. natural mutations occurring during cell division. The latter is a concern in isolating cells whose genomes will be sequenced, as accumulation of SNPs and genomic rearrangements and possibly horizontal transfer of mobile elements or loss of genomic islands in the enrichment broth will yield inaccurate genomic data about the cells in the environment. Such data may erroneously lead to assumptions on virulence factors, phylogeny, or origin of the strains. Essentially, the minimum amount of culturing steps should be used to obtain accurate results.

As *V. cholerae* can grow at a wide range of temperatures, several incubation temperatures in APW and TCBS, TTGA, or CA can be used for *V. cholerae* isolation. Incubating at the lower range of recommended temperatures (near 30°C) may enhance the growth of environmentally stressed cells exposed to low temperatures, but this may require longer incubation times on solid media. Incubating media at higher temperatures (> 37°C) may inhibit the growth of some non-*Vibrio* species but may also inhibit the growth of stressed *V. cholerae* cells. If possible, incubate media in parallel at multiple temperatures. Furthermore, stepwise increase in incubation temperature, 25°C (1–2hr) → 30°C (1–2hr) → 35°C can be used to sensitize the stressed cells to adapt and grow.

We strongly advise that a subculturing step onto non-selective media be utilized after isolation from TCBS, TTGA, or CA. This step serves two purposes. First, it is critical that pure colonies be isolated before proceeding to any genomic or phenotypic characterization

steps. Secondly, growth on TCBS is not suitable for the oxidase test, serotyping, or for direct PCR.

It is known that PCR amplification of target DNA in environmental samples can be inhibited by dissolved organics, such as humic acid. For that reason, we suggest performing a DNA extraction first. This typically adds only 3 to 4 hr to the protocol and results in high yield DNA with a decrease in inhibition during PCR amplification. For all direct PCR examinations (PCR on extracted or boiled APW), include the eubacterial PCR reaction to confirm template quality on samples. Further, we strongly suggest that direct PCR be done on 1:10, 1:100 dilutions (DNA template:TE buffer or DNA template: nuclease free water) in parallel with undiluted samples.

FISH allows visual quantification of all *V. cholerae* cells, regardless of their culturability, in a sample. One problem with this method is observation of auto-fluorescing constituents found in environmental water samples. All "positive" samples should be documented by digital or film photography, and confirmation by conventional PCR and/or Real-Time PCR of a parallel sample (frozen, but not fixed) is advisable.

The following table (Table 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. Please consult the troubleshooting sections from the referenced CPMB sections for further advice.

Anticipated Results— *V. cholerae* can be easily isolated from estuarine environments during the warm summer months, even in non-epidemic areas. Those chances decrease during the colder winter months, even from samples that are positive by PCR or DFA. Figure 3 shows the typical growth of *V. cholerae* on TCBS, TTGA, and CA media. Growth on TCBS by *V. cholerae* appears as small (1 to 3-mm diameter), flat, yellow colonies (Fig. 3). *V. cholerae* appear as medium to large (2 to 5-mm in diameter), flat, translucent colonies on TTGA (Fig. 3). 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. Growth on CA by *V. cholerae* appears as medium to large (2 to 5-mm in diameter) turquoise colonies. However, *V. cholerae* is a highly heterogeneous species can exhibit various colony morphologies including a rugose form (White 1940). Several different *V. cholerae* isolates should be used as positive controls as comparators for cultured cells. These selective media, TCBS, TTGA, and CA are all selective, but still allow the growth of other *Vibrio* species and related bacteria. For example, *V. parahaemolyticus* will appear as blue-green (occasionally yellow) colonies, *V. vulnificus* will appear as greenish yellow colonies, and *V. alginolyticus* as larger, sometimes swarming yellow colonies on TCBS. Therefore, colonies growing on these media are not necessarily all *V. cholerae,* hence they are labeled as presumptive until they can be confirmed by PCR. If several of these solid media are available to the investigator then it may be beneficial to "dot" or streak for isolation, presumptive *V. cholerae* colonies isolated from one media onto another that is selective for *V. cholerae* as well (TCBS → CA and TTGA, for instance). Visualizing the color and morphology of an isolate on one selective media that was recovered from a different selective media may help the investigator determine whether or not to proceed with PCR confirmation of that isolate.

Supplementary Table 1 lists a comprehensive, yet not exhaustive set of PCR primers used to characterize *V. cholerae* isolates. This table also lists the reaction temperatures and times as well as the expected amplicon size. Positive control strains known to encode the target region and yielding an appropriate size amplicon should be used for all PCR reactions.

Time Considerations—Choosing the appropriate methods discussed in this unit will determine how rapidly results can be achieved. Clinical diagnosis requires rapid determination of the etiological agent and rapid non-culturing techniques are currently being developed. In the case of cholera, symptoms are often distinguishable from other infections and stool typically requires no need for enrichment in APW and readily yield *V. cholerae* colonies on TCBS agar. Environmental monitoring often requires more time, depending on the method. However, PCR screening can be performed in 6–24 hours depending on the template, and DFA can be done in 4 hours or 24 hours if coupled with DVC. Use of multiple methods and genotypic characterization will significantly increase the amount of time needed to complete this work. In-depth genotypic characterization can be done in intervals after several rounds of sample collection and *V. cholerae* isolation. It is important to confirm any presumptive strain as being *V. cholerae* before time and resources are dedicated to genotypic characterization. As with any proposed work, a daily schedule with estimated timeframes may be helpful in efficiently accomplishing the investigators' goals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by the grants from National Science Foundation Grant 0813066, NIH Grant 2RO1A1039129-11A2, National Institutes of Health-Fogarty International Center Grant 1RC1TW008587-01, US Army Medical Research and Material Command Grant W81XWHO9201, NOAA Grant S0660009, and DHS Contract HSHQDC-10-C-00177.

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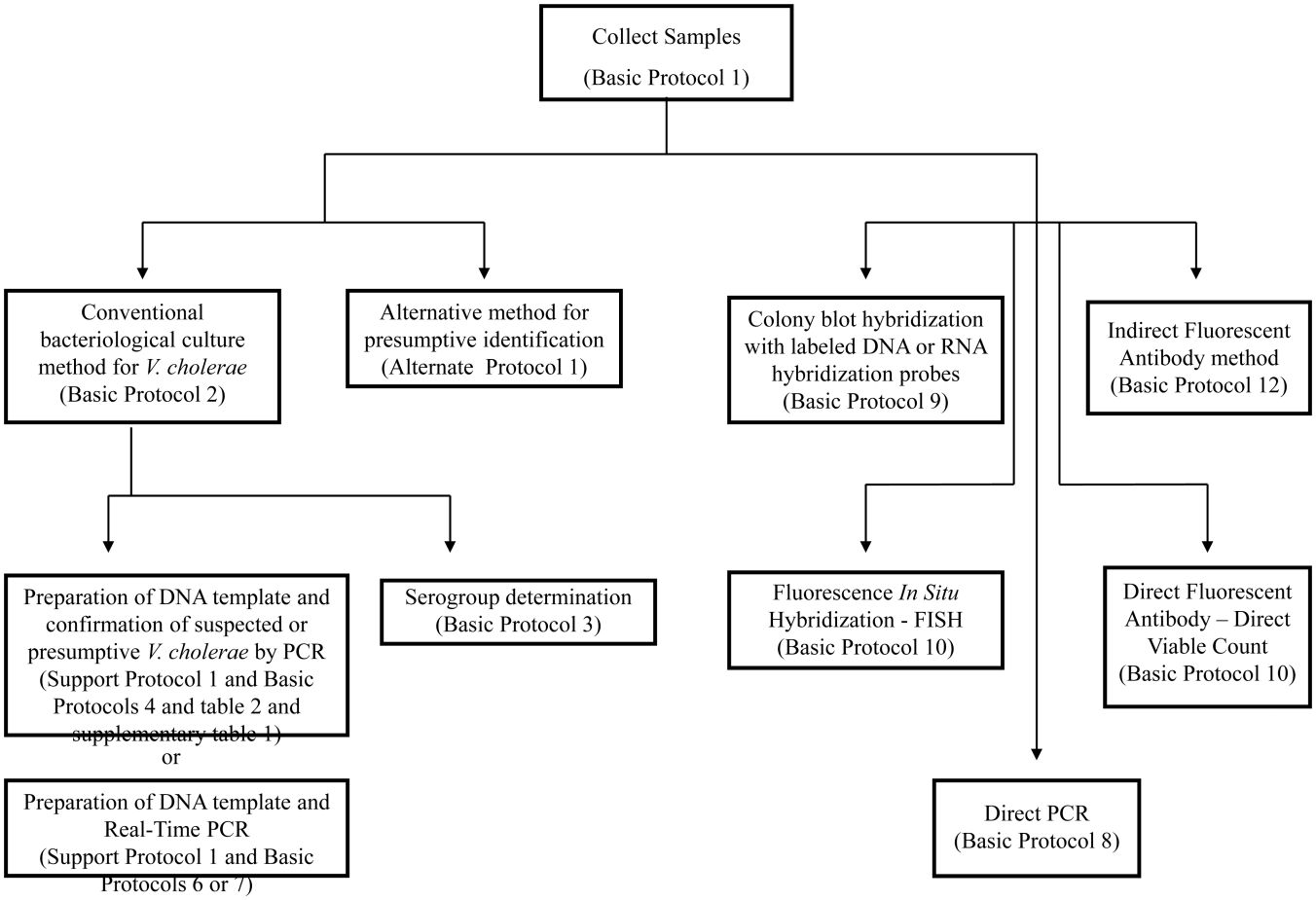

Figure 1.
Flowchart of methods (protocols) used to detect and/or isolate and characterize *V. cholerae* from the environment.

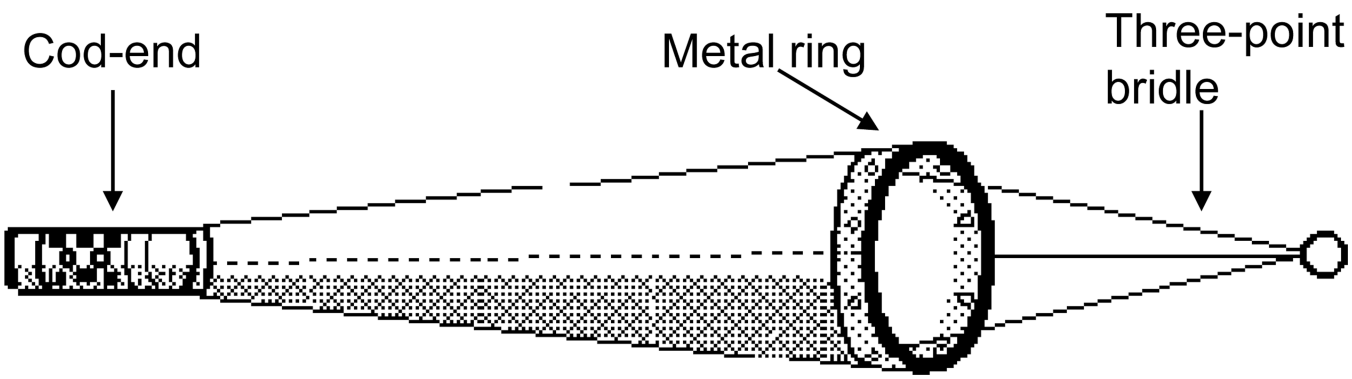

Figure 2.
Simple plankton net (Aquatic Research Instruments, Hope, ID, USA). The net is comprised of a metal ring and bridle, a heavy-duty nylon net of variable mesh size, and a PVC cod-end or collecting bucket which can be removed for easy sample collection. A flow meter may be mounted in the mouth of the net to the metal ring to measure volume when the net is towed.

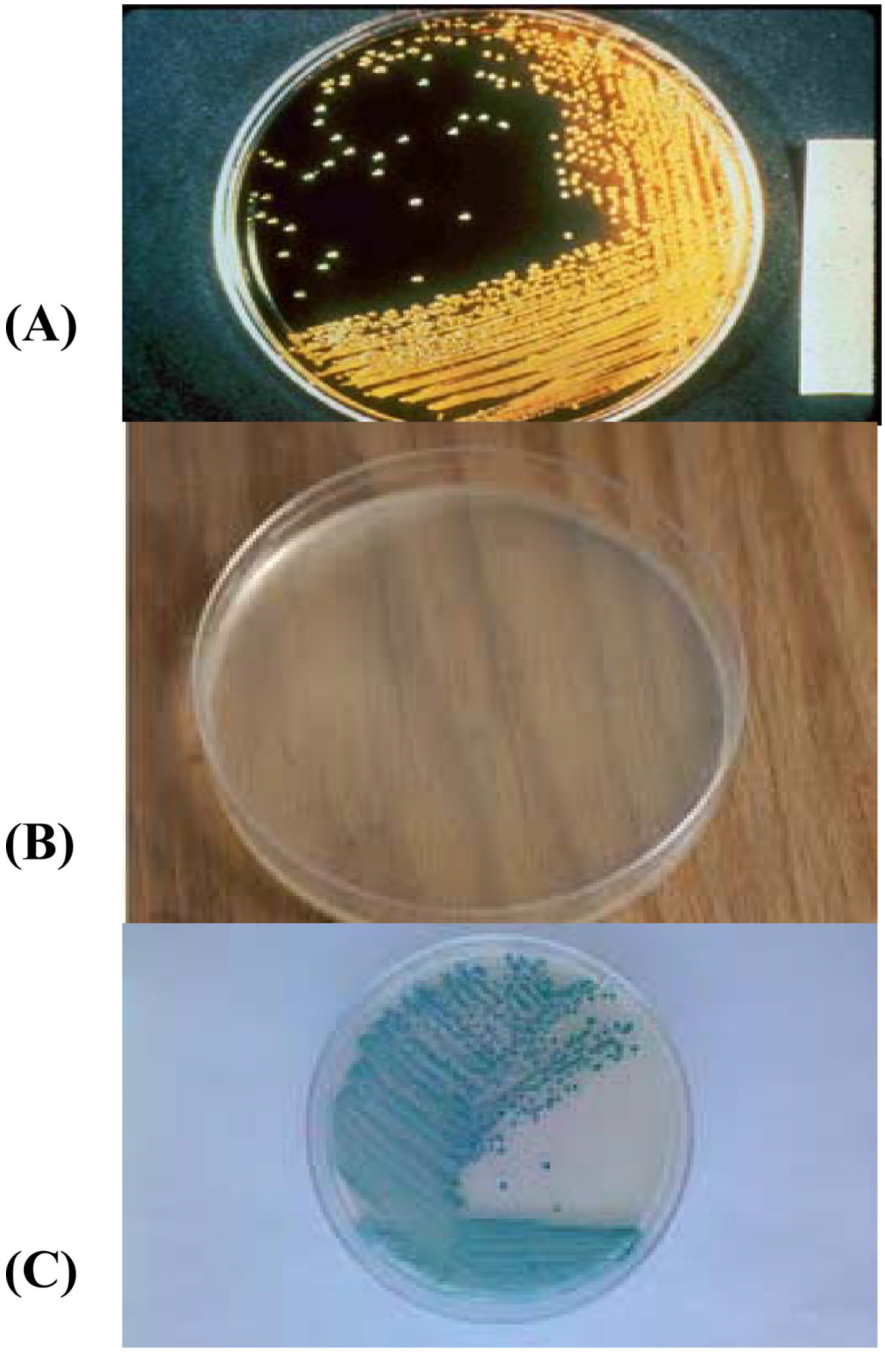

Figure 3.
Growth of *V. cholerae* O1 on (A) TCBS, (B) TTGA, (C) CHROMagar *Vibrio*, courtesy of Dr. Munir Alam, International Center for diarrheal Disease Research, Bangladesh. [*Color]

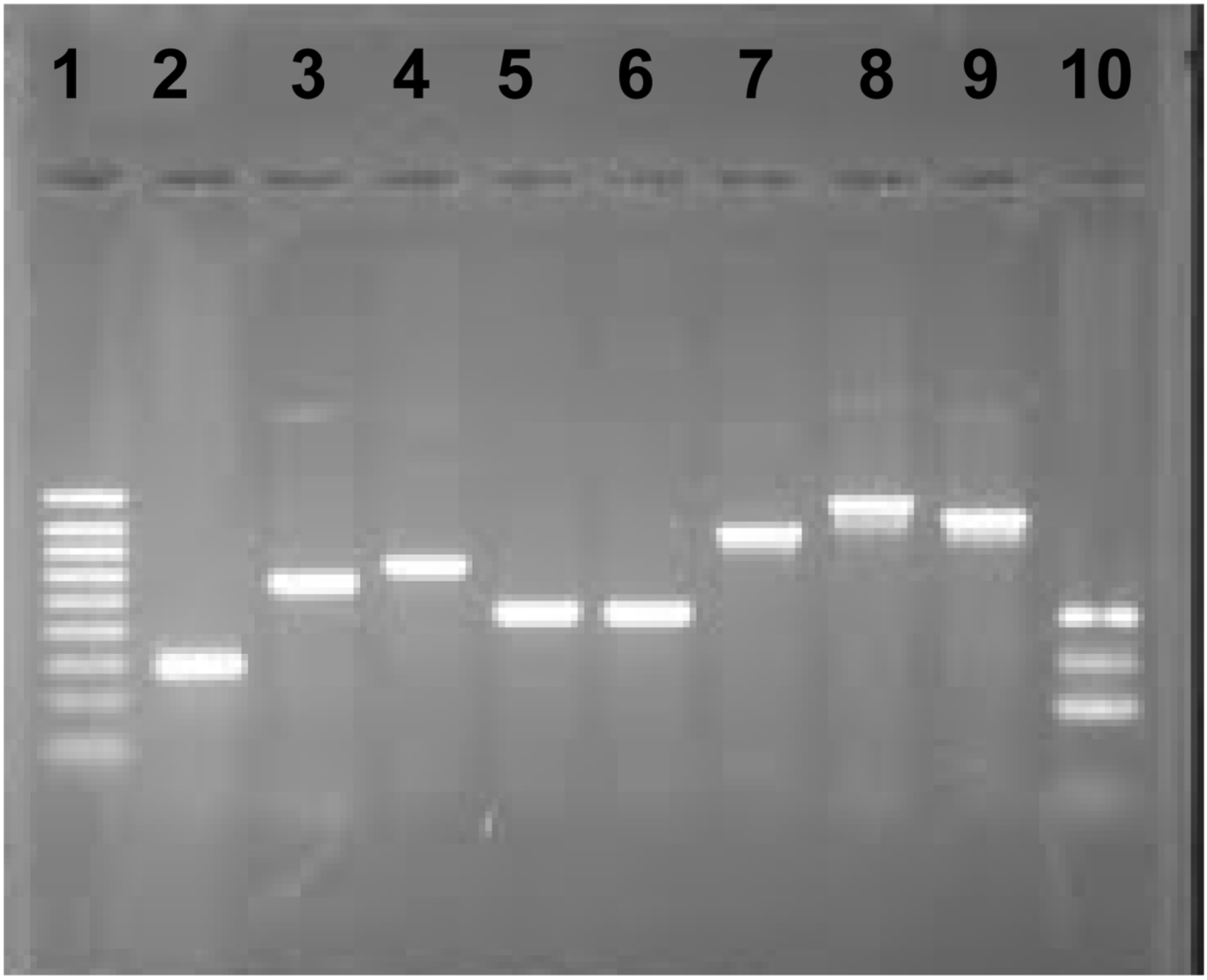

Figure 4.

Results 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.

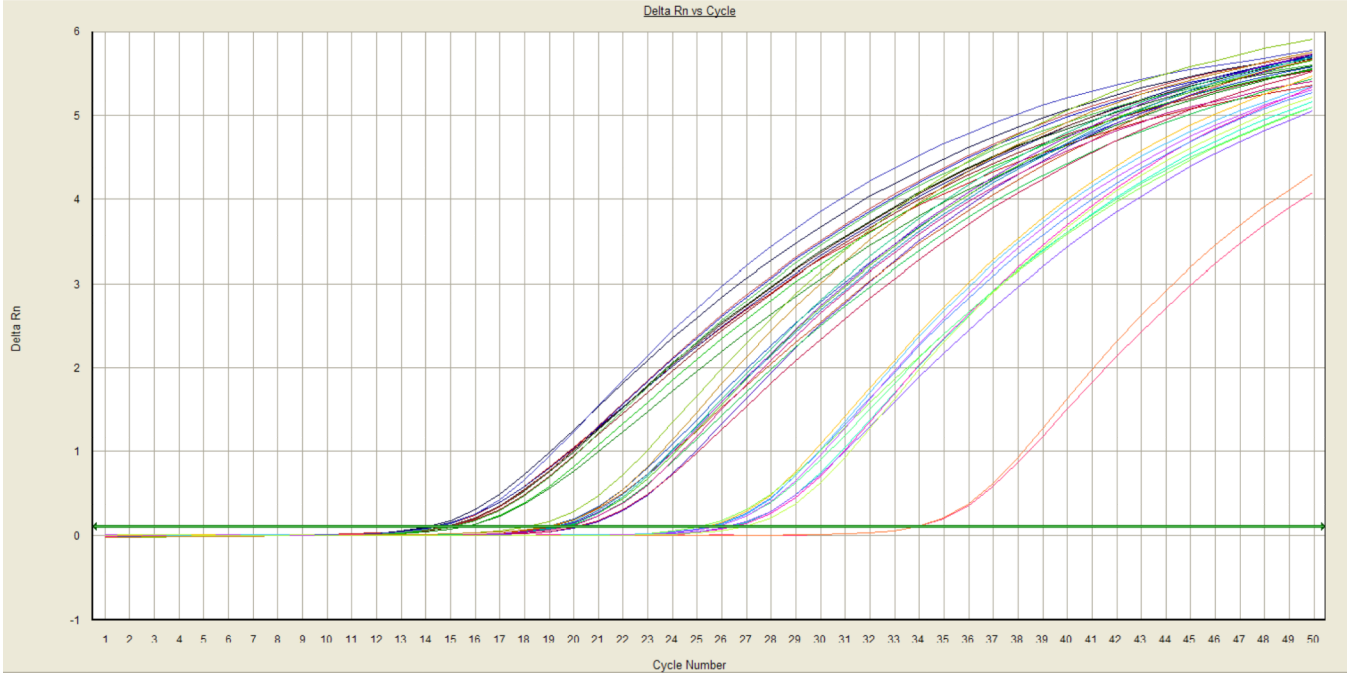

Figure 5.
Real-Time PCR amplification curves of *V. cholerae* spiked in fliter sterilized water. [*Color]

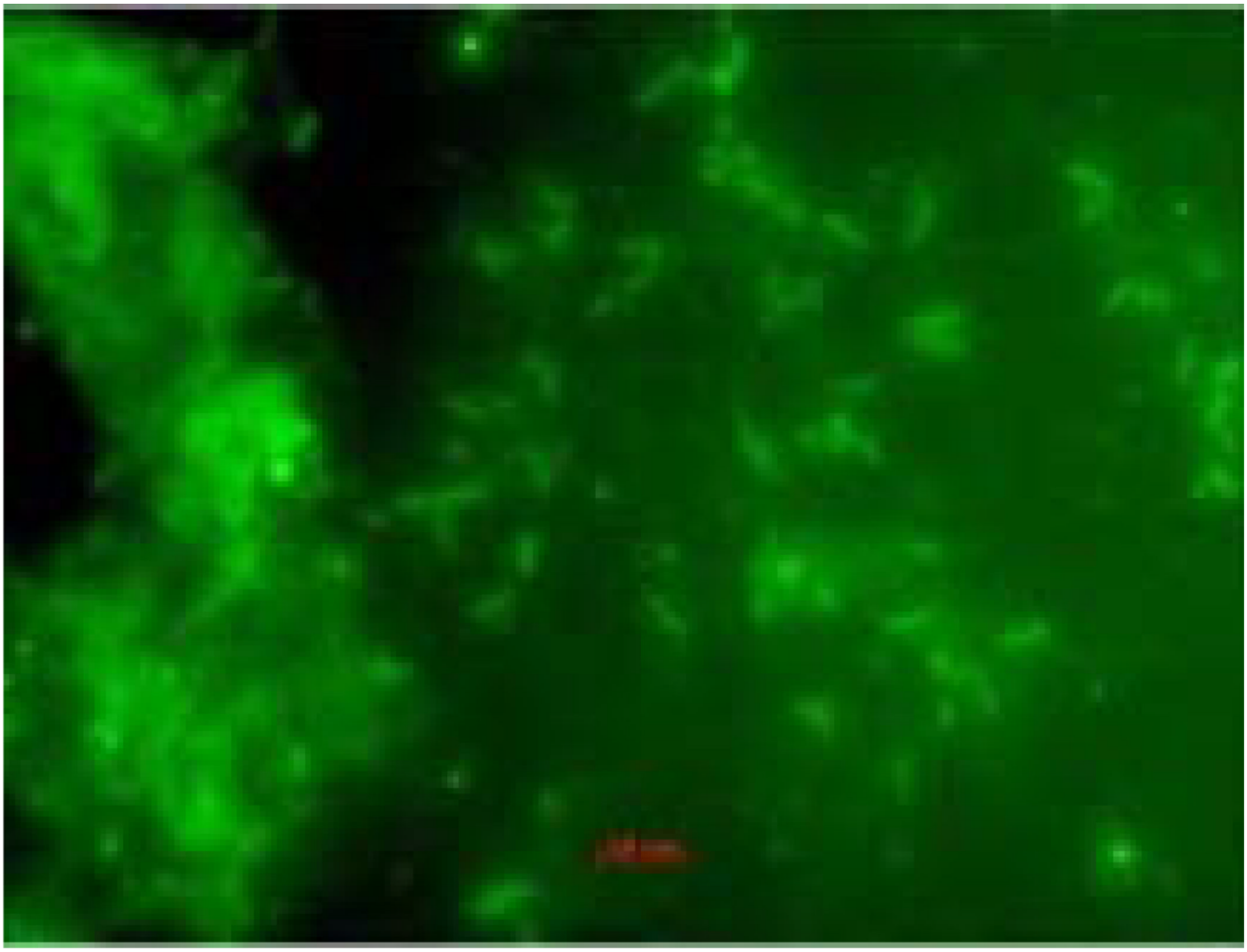

Figure 6.
Fluorescent *In-Situ* Hybridization (FISH) image of *V. cholerae* cells under epifluorescence microscopy. [*Color]

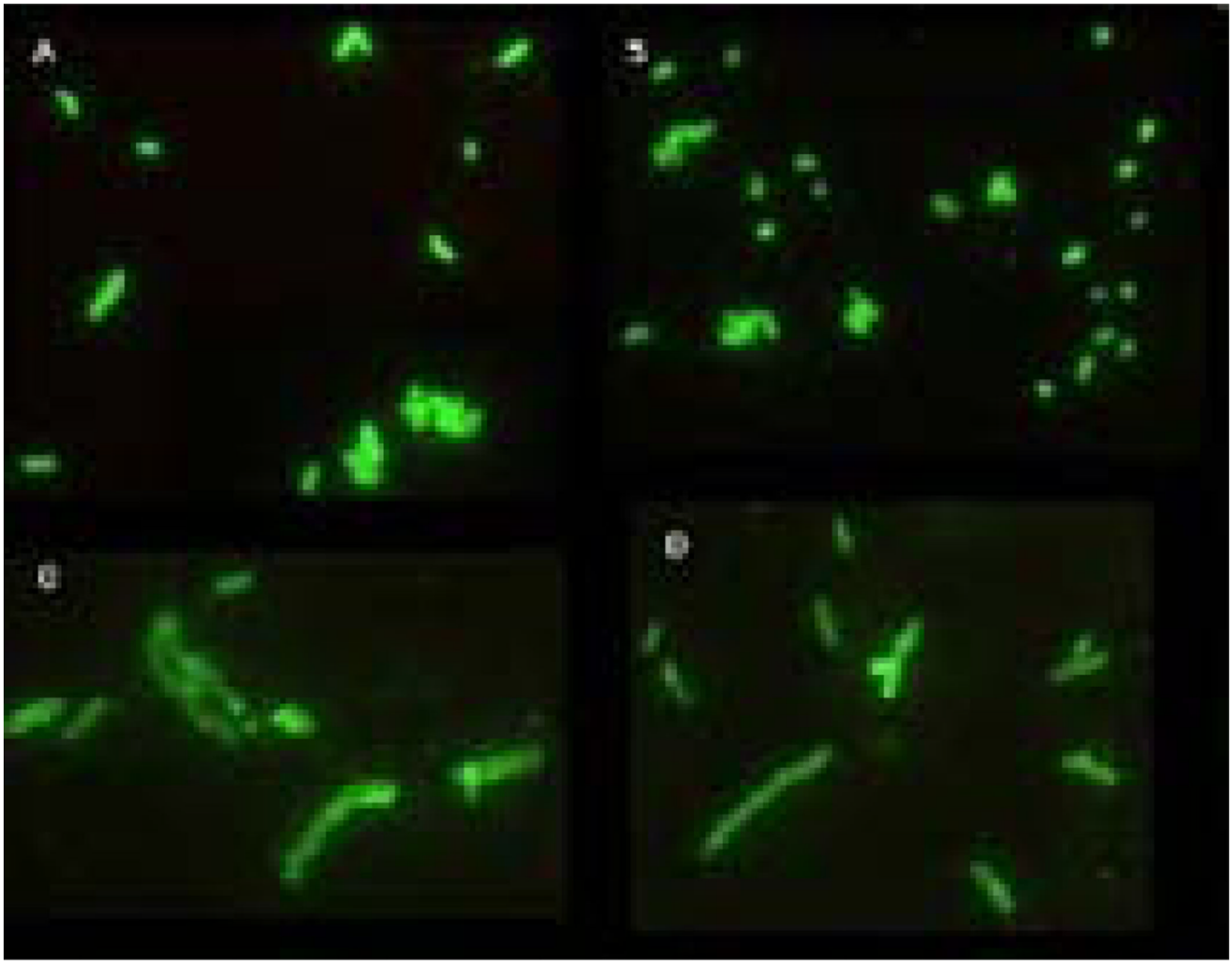

Figure 7.
DFA staining of *V. cholerae* O1 using the CholeraDFA Kit (New Horizon Diagnostics). (A) Fresh culture; (B) VBNC cells; (C) and (D), DVC-incubated cells. [*Color]

Table 1

Overview of Methods Presented in This Unit for Isolation and Detection of *V. cholerae*.

Protocol	Included methodology	Protocol ID
Isolation of presumptive *V. cholerae* and confirmation, using traditional methods	Specimen collection and transportation. Conventional bacteriological culture method for *V. cholerae*	Basic Protocol 1 Basic Protocol 2
	Alternate method for presumptive identification	Alternative Protocol 1
	Serogroup determination	Basic Protocol 3
Molecular methods for detection of *V. cholerae* Isolates	Preparation of DNA templates and confirmation of suspected or presumptive *V. cholerae* by PCR	Support Protocol 1 and Basic Protocol 4
	Real Time PCR	Basic Protocols 6 and 7
	Colony blot hybridization with labeled DNA or RNA hybridization probes	Basic Protocol 9
Molecular methods for direct detection of *V. cholerae* in environmental samples	Fluorescence *In Situ* Hybridization (FISH) Direct PCR	Basic Protocol 10 Basic Protocol 8
Direct detection and/or enumeration of *V. cholerae* using immunological methods	Direct Fluorescent Antibody – Direct Viable Count (DFA-DVC)	Basic Protocol 11
	Indirect Fluorescent Antibody (IFA) method	Basic Protocol 12

Table 2

V. cholerae positive control strains.

Strain	Serogroup	Serotype	Biotype	Source	Year of Isolation
V. cholerae NCTC 8457	O1	Inaba	El Tor	Clinical case (pilgrim en route to Mecca) from a quarantine station in El Tor (Al-Tur), Egypt	1930
V. cholerae MAK 757	O1	Ogawa	El Tor	Clinical case from Makassar, Celebes Islands, a coastal region (now Sulawesi, Indonesia)	1937
V. cholerae N16961	O1	Inaba	El Tor	Clinical case from Bangladesh	1975
V. cholerae 2010EL-1786	O1	Ogawa	El Tor	Clinical case from Artibonite Department, Haiti	2010
V. cholerae NCTC 569B	O1	Inaba	Classical	Clinical case in India	1948
V. cholerae O395	O1	Ogawa	Classical	Clinical case from India	1964
V. cholerae LMG 4406	non-O1/non-O139	NA	Albensis	Fish isolate from the Elbe River, Germany	1896
V. cholerae 26	non-O1/non-O139	NA	NA	Environmental isolate from Santa Cruz, CA, USA (Pacific Ocean)	1983
V. cholerae MO45	O139	NA	NA	Clinical isolate from Madras, India	1992

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Table 3

PCR targets and primers for *V. cholerae*.

Target	Primer	Sequence (5'-3')	Amplicon	Ref.
ITS	pVC-F2	TTAAGCSTTTTCRCTGAGAATG	295-310	(Chun, Huq et al. 1999)
	PVCM-R1	AGTCACTTAACCATACAACCCG		
ctxA	PCTA-94F	CGGGCAGATTCTAGACCTCCTG	563	(Fields, Popovic et al. 1992)
	PCTA-614R	CGATGATCTTGGAGCATTCCCAC		
toxR	pToxR-101F	CCTTCGATCCCCTAAGCAATAC	778	(Rivera, Chun et al. 2001)
	pToxR-837R	AGGGTTAGCAACCGATGCGTAAG		
tcpA	pTcpA-72F	CACGATAAGAAAACCGGTCAAGAG	452	(Keasler and Hall 1993)
	pTcpAET-477R	CGAAAGCACCTTCTTTCACGTTG	621	
	pTcpACL-647R	TTACCAAATGCAACGCCGAATG		
zot	PZot-225F	TCGCTTAACGATGGCGCGTTTT	946	(Rivera, Chun et al. 2001)
	PZot-1129R	AACCCCGTTTCACTTCTACCCA		
ompU	pOmpU-80F	ACGCTGACGGAATCAACCAAAG	868	(Rivera, Chun et al. 2001)
	pOmpU-906R	GCGGAAGTTTGGCTTGAAGTAG		
ctxA	VCT1	ACAGAGTGAGTACTTTGACC	308	(Hoshino, Yamasaki et al. 1998)
	VCT2	ATACCATCCATATATTTGGGAG		
O1-*rfb*	O1F2-1	GTTTCACTGAACAGATGGG	192	(Hoshino, Yamasaki et al. 1998)
	O1R2-2	GGTCATCTGTAAGTACAAC		
O139-*rfb*	O139F2	AGCCTCTTTATTACGGGTGG	449	(Hoshino, Yamasaki et al. 1998)
	O139R2	GTCAAACCCGATCGTAAAGG		
ompW	ompW-F	CACCAAGAAGGTGACTTTATTGTG	588	(Nandi, Nandy et al. 2000)
	ompW-R	GAACTTATAACCACCCGCG		
ctxA	CtxA-F	CTCAGACGGGATTTGTTAGGCACG	302	(Shirai, Nishibuchi et al. 1991; Nandi, Nandy et al. 2000)
	CtxA-R	TCTATCTCTGTAGCCCCTATTACG		
16S rDNA[a]	16S-F 16S-R	CAGCMGCCGCGGTAATWC ACGGGCGGTGTGTRC	888	(Amann, Ludweg et al. 1995)

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

Table 4

Real-Time PCR primers and probes.

Primer/Probe/Beacon Name	Sequence	Ref
hylA-probe	FAM-TCAACCGATGCGATTGCCCAAGA-TAMRA [1]	(Lyon, 2001)
hylA-F-primer	TGCGTTAAACACGAAGCGAT	
hylA-R-primer	AAGTCTTACATTGTGCTTGGGTCA	
MBrtxA	CGCGATCACCAGAGCGCCAAGAAGTGACTCGTAGATCGCG [2]	(Gubala and Proll, 2006)
MBepsM	CGCGATGCCACCGACATCGTAACGCTCCGATCGCG [3]	
MBompW	CCGAAGAAACAACGGCAACCTACAAAGCTTCGG [4]	
MBtcpA	CGCGACGCTGAAACCTTACCAAGGCTGACCAAGTCGCG [5]	

[1] The TaqMan *V. cholerae*-specific probe is an oligonucleotide with a 5'reporter dye (FAM-6-carbooxyfluorescein) and a 3'quencher dye (TAMRA-6-carboxy-N'N'N'N'-tetramethylrhodamine).

[2] FAM (6-carboxyfluorescein) fluorophore and Dabcyl quencher

[3] Texas Red fluorophore and BHQ2 quencher

[4] Cy5 fluorophore and BHQ3 quencher

[5] Cy3 fluorophore and BHQ2 quencher

Table 5

Common problems that may be experienced in performing the basic and alternate protocols from this unit

Basic Protocol 1	
Little or no plankton in cod-end collecting bucket	Check pore size of plankton net and ensure that it is 64 mm. Zooplankton should be sampled near dawn or dusk (1-2 hr after sunrise or before sunset) when they are nearer to the surface. Filter more water through plankton net.
Basic Protocol 2	
No *V. cholerae* growth	Adjust dilution volumes and/or incobation temperature. Incubate petri dishes at several temperatures
V. cholerae overgrowth	Adjust dilutions or use a smaller volume loop to spread onto agar
Basic Protocol 3	
Autoagglutination or clumping in saline without antisera	“Rough” morphotypes cannot be serogrouped with anitsera. Use O1/O139 *rfb* PCR primers with Basic Protocol 4 to test for toxigenic serogroups.
Support Protocol 1	
Low DNA concentration	Increase volume of boiled cells
Basic Protocol 4	
No PCR product with positive control	Ensure all components are added to reaction at the proper concentration. Use fresh dNTPs. Prepare fresh crude template of positive control as it will degrade over time. Dilute crude template 1:5000 or more and repeat the control reaction. Quantify crude template by gel electrophoresis (~10 ml) to ensure sufficient template concentration.
PCR product with negative control	Most likely caused by carry-over contamination in one of the reaction components. Make new components.
Basic Protocols 6 and 7	
Weak amplification with positive control	Ensure all components are added to reaction at the proper concentration. Use fresh dNTPs. Prepare fresh crude template of positive control as it will degrade over time. Dilute crude template 1:5000 or more and repeat the control reaction. Quantify crude template by gel electrophoresis (~10 ml) to ensure sufficient template concentration.
Strong amplification with negative control	Most likely caused by carry-over contamination in one of the reaction components. Make new components.
Basic Protocol 8	
Positive control is negative	Ensure solutions used are RNase-free.

Basic Protocol 1	
	Increase incubation time of lysis step (10% SDS), especially if colonies are larger than 3 mm. Ensure that the correct microscope filter is used for fluorochrome selected. (Other fluorochromes may be used.)
Positive control gives weak signal	Check scanning settings on detection instrument. Increase probe concentration and/or hybridization time.
Alternate Protocol 3	
Positive control is negative or weak	Ensure that solutions are used in correct order. Overexposure to UV source will degrade DNA template. Consider using positively charged nylon membranes, which do not need crosslinking. Extend hybridization time. Extend development time.
Positive control is overdeveloped or background is high	Check hybridization temperature. Do not allow membrane to dry. Decrease development time.
Basic Protocol 9	
Positive control from Alternate Protocol 3 is negative or weak	Check efficiency of probe labeling reaction. Increase probe concentration (≤ 25 ng/ml)
Basic Protocol 10	
Weak Fluorescence	Increase probe concentration Check hybridization temperature Increase hybridization time
Nonspecific Staining	Perform more stringent wash step
Basic Protocol 11	
No PCR product with positive control	Ensure all components are added to reaction at the proper concentration. Use fresh dNTPs. Prepare fresh crude template of positive control as it will degrade over time. Dilute crude template 1:5000 or more and repeat the control reaction. Quantify crude template by gel electrophoresis (~10 ml) to ensure sufficient template concentration.
PCR product with negative control	Most likely caused by carry-over contamination in one of the reaction components. Make new components.
Basic Protocol 11	
Positive control is negative	Ensure that the proper filter set is used on fluorescent microscope. Bengal DFA (*V. cholerae* O139) kit positive control is sometimes poor. Prepare positive control from laboratory reference strain.
Basic Protocol 12	

Basic Protocol 1	
Positive control signal is weak	Increase amount of *V. cholerae* O1 antiserum and FITC conjugate.