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Genetic Screens and Biochemical Assays to Characterize *Vibrio cholerae* O1 Biotypes: Classical and El Tor

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Abstract

Vibrio cholerae serogroup O1 has two biotypes, classical and El Tor, the latter of which has displaced the prior and has been the causative agent for the ongoing seventh pandemic. However, reports since 2001 have identified clinical isolates of El Tor that have classical O1 biotype genetic and phenotypic characteristics. These El Tor variants have been emerging in clinical settings with increased frequency, including the 2010 cholera outbreak in Haiti. The emergence of El Tor variants warrants the proper and timely identification of clinical (or environmental) isolates' biotype. This unit describes some quick and simple genetic screens and phenotypic assays (biochemical characterization), to be performed simultaneously, commonly used to distinguish biotype and initiate characterization of any clinical (or environmental) isolates of *Vibrio cholerae* O1.

Keywords

Vibrio cholerae O1; Classical biotype; El Tor biotype; Genetic screens; Biochemical characterization

Introduction

The proper and timely identification of the causative agent of any disease is paramount in the treatment of not only the symptoms of the disease, but the disease itself. The etiological agent of cholera is the bacterium *Vibrio cholerae*, which can be categorized to multiple serogroups. Of these, two serogroups, O1 and O139, have been known to or have the potential to cause cholera epidemics. Furthermore, the O1 serogroup can be further divided into two biotypes, classical and El Tor. Although the importance of distinguishing the biotype of the infecting agent does not immediately impact the treatment for cholera, which is similar regardless of the infecting strain, proper identification is required not only for the characterization of the outbreaks for epidemiological studies, such as those reported from the recent 2010 outbreak in Haiti, but also for basic research purposes, where genetic manipulation is biotype dependent. Unfortunately, evolution of the bacteria has resulted in the emergence of more serogroups and even variations within medically relevant serogroups, particularly the O1 serogroup. This has led to a complex identification system, where a strain of one biotype has obtained some characteristics of another biotype, rendering single assay identification of strains unreliable, such that it is desirable to apply multiple assays.

This set of protocols details some of the different genetic and phenotypic assays commonly utilized when characterizing *Vibrio cholerae* clinical or environmental isolates (Lan and Reeves 2002; Nair et al. 2002; Ansaruzzaman et al. 2004; Nair et al. 2006a; Nair et al. 2006b; Safa et al. 2006; Ansaruzzaman et al. 2007). Basic Protocol 1 details a genetic screen commonly used to examine the sequences of the cholera toxin B subunit (*ctxB*) (Nair et al. 2006a; Morita et al. 2008; Chatterjee et al. 2009) and the major pilin protein TcpA (*tcpA*) (Safa et al. 2008; Chatterjee et al. 2009). Cholera toxin, composed of the A and B subunits, is the major virulence factor that is directly responsible for the rice-water diarrhea associated with cholera. TcpA is the major pilin protein that makes up the pilus structure, another major virulence factor expressed by *V. cholerae* O1 under the proper conditions. This cell surface structure allows the bacterium to attach to the intestinal walls and establish a colony. The sequences of these genes, *ctxB* and *tcpA*, are completely conserved within each of the two biotypes, but differ in a few bases across the biotypes.

The remaining assays, polymyxin B resistance, citrate metabolism, proteolysis, hemolysis, motility, Voges-Proskauer and biofilm production (Basic Protocols 2 through 8), are phenotypic assays that compare the phenotypes of the isolates to O395, a wild-type classical biotype strain, and N16961 and C6706, two wild-type El Tor biotypes.

Classical O1 biotype strains generally show sensitivity to polymyxin B. Polymyxin B is a peptide antibiotic that disrupts the outer membrane in gram negative bacteria, rendering the cells especially susceptible to lysis. El Tor is resistant to polymyxin B.

Interestingly, the ability of the two biotypes to grow on citrate as a sole carbon source differs. In the case of the classical biotype, in particular wild-type O395, citrate as a sole carbon source cannot support growth, whereas the El Tor biotype strains, N16961 and C6706, are able to utilize citrate to support growth.

The proteolysis assay performed on milk agar plates, is suggestive of HapR regulated protease activity. HapR is a regulator that regulates many processes, including expression of proteases that cleave the casein protein found in the milk agar plates, resulting in a zone of clearance around the bacterial growth. Wild-type classical O395 has a deleted base in the *hapR* gene, resulting in a truncated HapR, and therefore is phenotypically HapR negative. Wild-type El Tor C6706, which codes for a functional HapR, results in a zone of clearance around the bacterial growth and is therefore phenotypically HapR positive. Interestingly, wild-type N16961, an El Tor biotype, also codes for a truncated *hapR*, with the same deleted base as classical O395 and is therefore phenotypically HapR negative. Therefore it is important to compare proteolysis of the isolates to classical O395 and either El Tor C6706 or C6706 and N16961.

The degree of hemolysis can vary among the El Tor variants as demonstrated by spotted colonies on blood agar plates but can be scored as positive (hemolytic) or negative (non-hemolytic). The observed hemolysis is the result of the bacteria secreting hemolytic enzymes that lyse the red blood cells in the agar, resulting in a clearing or lysis zone around the colony. Wild-type El Tor N16961 (and C6706) is hemolytic as indicated by a zone of red blood cell clearance (lysis), whereas wild-type classical O395 is non-hemolytic.

Motility of the isolates can be compared to the motility of classical O395 and El Tor N16961 (or C6706). Classical O395 has been observed to be less motile than El Tor N16961 (and C6706) at 37°C.

The Voges-Proskauer (VP) assay tests the ability of the bacteria to utilize glucose as a carbon source to produce the end product of acetoin via fermentation (Blazevic and Ederer 1975). Acetoin, in the presence of the reagents potassium hydroxide and alpha-naphthol (α -naphthol) turns a bright pink, as is the case with wild-type El Tor N16961 (and C6706). Classical biotype wild-type strain O395 does not produce a bright pink color, but rather has no color change or produces a faint pink color, indicative of a negative VP test, suggesting little or no acetoin is produced.

Although each assay is designed to distinguish an isolate as either classical or El Tor biotype, it must be noted that conducting as many assays as possible in conjunction and relation to each other is highly recommended prior to concluding a particular isolate's true biotype. In fact, differences between the two wild-type El Tor biotypes, N16961 and C6706 are also observed in some of the different assays. However, the importance of multiple assays has been emphasized in practice (Lan and Reeves 2002; Nair et al. 2002; Ansaruzzaman et al. 2004; Nusrin et al. 2004; Lee et al. 2006; Nair et al. 2006a; Nair et al. 2006b; Safa et al. 2006; Ansaruzzaman et al. 2007; Morita et al. 2008; Nair et al. 2008; Chatterjee et al. 2009; Nguyen et al. 2009), especially in light of a series of reports starting in 2001 that documented clinical isolates as having an El Tor biotype background, but exhibiting either genotypic, phenotypic or both genotypic and phenotypic classical traits (Lan and Reeves 2002; Nair et al. 2002; Ansaruzzaman et al. 2004; Nusrin et al. 2004; Lee et al. 2006; Nair et al. 2006a; Nair et al. 2006b; Safa et al. 2006; Ansaruzzaman et al. 2007; Morita et al. 2008; Nair et al. 2008; Chatterjee et al. 2009; Nguyen et al. 2009). These particular isolates have been since dubbed El Tor variants (Nair et al. 2002; Nusrin et al. 2004) and emphasize the importance of conducting as many assays as possible to fully characterize and determine the identity of an isolate(s).

Basic Protocol 1 – PCR Based Genetic Screens Using *tcpA* and *ctxB*

This protocol requires purified chromosomal DNA, and involves the polymerase chain reaction (PCR) amplification of the two genes *tcpA* and *ctxB*, followed by sequencing of the PCR products to determine the exact sequences of the genes. The sequences of the genes are conserved within but differ across the two biotypes, and therefore comparison of the gene sequences from the isolate(s) to those of classical O395 or El Tor N16961 (or C6706), will help characterize the isolate(s). Single colonies of the strains of interest are inoculated into rich media grown overnight (12 to 15 hours) and chromosomal DNA purified from these cultures. It is strongly recommended to include classical O395, El Tor N16961 and El Tor C6706 as controls for the PCR, but are not required for sequencing, as they are available on the NCBI website. The sequence for C6706 is not available online, and can be sequenced alongside the strains of interest.

Materials

Overnight Cultures

QIAprep Spin Miniprep Kit (Qiagen Cat. #27104)

NanoDrop ND100 Spectrophotometer (NanoDrop Model #ND100)

200 µl Sterile Thin-walled PCR Tubes (Corning Cat. #6571)

tcpA/ctxB Forward and Reverse Primers

1.25 mM dNTP Mix (NEB Cat. #N0446S) (see recipe)

Pfu Turbo Cx HotStart DNA Polymerase (Agilent Cat. #600412-51)

10X *Pfu* Turbo Cx Reaction Buffer (Stratagene Cat. #600410-52)

PCR Thermal Cycler (to hold 200 µl PCR tubes) with Heated Lid

UltraPure Agarose (Invitrogen Cat. #16500-500)

1X TBE (see recipe)

6X DNA Gel Loading Dye (see recipe)

QIAquick PCR Purification Kit (Qiagen Cat. #28104)

1. Design primers to anneal approximately 50-75 bp upstream and downstream of the translational start and stop sites, respectively, of either *tcpA* or *ctxB*.
 - a. Alternatively, the primers listed in Table 1 for both *tcpA* and *ctxB* have been used successfully.
2. Grow up cultures and isolate chromosomal DNA using established methods/kits (QIAprep Spin Miniprep Kit).
 - a. Resuspend the DNA in the resuspension buffer (EB Buffer) provided in the kit.
 - b. Ensure high quality, clean DNA using the NanoDrop ND100 spectrophotometer using the resuspension buffer (EB Buffer) as a blank. High quality, clean DNA should yield an A260/280 between 1.8 and 2.0.
3. Simultaneously, quantitate the amount of DNA using the NanoDrop ND100 spectrophotometer, using the resuspension buffer (EB Buffer) as a blank.
 - a. From a 2 ml overnight culture, the DNA resuspended in 300 µl of EB buffer can be expected to yield a concentration of 100 – 200 ng/µl, with an A260/280 around 1.87
4. Prepare the following PCR reaction mix on ice in a 200 µl thin-walled PCR tube:
5. Amplify using the following PCR protocol:

- i. 30 sec at 98°C
 - ii. 34 cycles of:
 - a. 45 sec at 98 °C
 - b. 30 sec at 60 °C
 - c. 90 sec at 72 °C
 - iii. 10 min at 72 °C
 - iv. Hold at 4 °C until ready to proceed with next step.
6. Verify positive PCR products by running a small aliquot (5 µl) of each reaction on a 1% agarose gel in 1X TBE buffer as per standard protocols.
 - a. To minimize the amount of PCR run on a gel for verification purposes, mix 5 µl of PCR reaction, 5 µl of sterile double-distilled water and 2 µl of 6x DNA gel loading dye.
7. Purify the PCR products using the QIAquick PCR purification kit following the manufacturer's recommendations.
8. Send off for sequencing using the same primers used in the PCR reaction.
9. Compare the sequences to the published sequences available on the NCBI website
 - a. Go to the NCBI website for genes (<http://www.ncbi.nlm.nih.gov/gene/>) and search the following accession numbers. Type the **bold-faced** accession numbers in the search query box. When conducting your gene sequence analysis, you will need to click the link to the FASTA format of the gene sequence.
 - i. *tcpA* – in classical: **VC0395_A0353**; in El Tor: **VC_0828**
 - ii. *ctxB* – in classical: the region between **VC0395_A1059** to **VC0395_A1060**; in El Tor: **VC_1456**

Basic Protocol 2 – Polymyxin B Resistance Assay

This assay allows you to determine whether the isolate(s) of interest has a polymyxin B resistance profile similar to that observed in El Tor biotypes, or if the isolate(s) is sensitive to this peptide antibiotic, such as the classical biotype. This procedure is straightforward and requires the streaking for single colonies of the different isolates and controls (wild-type classical and El Tor strains) on rich media plates containing polymyxin B (50 IU/ml). Resistance is determined by visual inspection for the growth of single colonies after incubation at 37°C overnight (12 to 15 hours).

Materials

Luria-Bertani (LB) Agar Plates (Standard size of 100 mm X 15 mm)

Polymyxin B (SigmaAldrich Cat. #P1004-100MU)

Sterile Flat Toothpicks

37°C Incubator

- Make LB agar plates containing 50 IU/ μ l polymyxin B.
 - i. Polymyxin B can be dissolved in sterile double-distilled water to a concentration of 50,000 IU/ μ l.
 - ii. Autoclave the LB agar media as per standard protocols and add the polymyxin B to the correct final concentration (50 IU/ μ l) once the autoclaved LB agar media has cooled enough to be handled without discomfort, just prior to pouring the plates. Adding the polymyxin B when the LB agar is too hot will render the drug inactive.
- Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - i. Be sure to include at least the classical wild-type strain O395 and both El Tor wild-type strains N16961 and C6706.
- Divide the bottom of the plate into sections using a marker.
 - i. The number of sections will depend on the number of isolates being tested. You should plan to include 2 extra sections to include to classical wild-type strain (O395) and the El Tor wild-type strain (N16961 or C6706) as polymyxin B sensitive and resistant controls, respectively.
- From single overnight colonies, streak out each strain in one section of the plate for single colonies.
 - i. Depending on the size of each section, care should be taken not to cross-contaminate between the strains.
 - ii. Streaks should be made for single colonies so that heavy streaks do not result in too high a density of cells for the antibiotic to be effective. Keep in mind that polymyxin B, like all antibiotics, have an effective concentration that is also cell density dependent.
- Incubate the plates upside down overnight at 37°C.
 - i. El Tor biotype strains are polymyxin B resistant, whereas classical biotype strains are sensitive.

Basic Protocol 3 – Citrate Metabolism

Minimal media supplemented with citrate as a sole carbon source can be used to distinguish between the classical and El Tor biotypes. Streaking out or patching single colonies of El Tor biotype strains onto minimal citrate media plates and incubating them overnight (12 to 15 hours) at 37°C is sufficient to support growth. However, the wild-type classical biotype strain O395, cannot grow when supplied with citrate as a sole carbon source, and thus this quick assay lends further evidence to characterize an isolate(s) as either El Tor or classical biotype background.

Materials

Luria-Bertani (LB) Agar Plates (Standard size of 100 mm X 15 mm)

Minimal Citrate Media Plates (see recipe) (Standard size of 100 mm X 15 mm)

Sterile Flat Toothpicks

Patching Grid (see Figure 1)

37°C Incubator

1. Make minimal media plates containing citrate as a sole carbon source (see Reagents and Solutions for recipe).
2. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to streak out the wild type control strains, classical O395 and El Tor N16961 (or C6706).
3. Patch single colonies from the rich media plate to the minimal citrate media plate using flat ended toothpicks. Using a pre-fabricated grid (Figure 1) is very useful for this procedure.
4. Incubate the plates upside down overnight at 37°C.
 - a. Classical O395 is unable to utilize citrate as a sole carbon source and therefore will not show any growth on these plates.
 - b. El Tor N16961 (and C6706) can utilize citrate as a sole carbon source and therefore the patches will show overnight growth.

Basic Protocol 4 – Casein Hydrolysis Protease Assay on Milk Agar Plates

The casein hydrolysis protease assay determines whether a *Vibrio* strain has the ability to produce and secrete functional proteases that can cleave the milk protein, casein, found in the milk agar plates. This protocol involves streaking or patching the strains of interest on milk agar plates, incubating them overnight (12 to 15 hours) at 37°C and observing for a zone of clearance around the bacterial growth. A positive result, or a visible zone of clearance, is an indication that the strain has a functional HapR regulator coded by *hapR*, as observed in C6706, and is commonly referred to as HapR positive. A negative result of no clearing may be the result of a deleted base in *hapR* giving rise to a non-functional truncated HapR, as seen in classical O395 or El Tor N16961, or may be the result of a different mutation(s) and may require further investigation.

Materials

Milk Agar Plates (see recipe) (Standard size of 100 mm X 15 mm)

Sterile Flat Toothpicks

37°C Incubator

1. Make milk agar plates (see Reagents and Solutions for recipe).
2. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to include at least the classical wild-type strain O395 and both El Tor wild-type strains N16961 and C6706.
3. Divide the bottom of the plate into sections using a marker.
 - a. The number of sections will depend on the number of isolates being tested. You should plan to include at least 2 extra sections to include to classical wild-type strain (O395) and the El Tor wild-type strains N16961 and C6706. Although N16961 and C6706 are both El Tor wild-type strains, N16961 is *hapR*⁻ and will therefore test negative (no casein hydrolysis or clearing) on the milk plate.
4. From single overnight colonies, streak out each strain in one section of the plate for single colonies.
 - a. Depending on the size of each section, care should be taken not to cross-contaminate between the strains.
 - b. Streak the single colony using the wide end of flat toothpicks in a single motion from the outer perimeter towards the center of the plate (Figure 2).
5. Incubate the plates upside down overnight at 37°C.

Basic Protocol 5 – Hemolysis Assay on Blood Agar Plates

Hemolytic activity of the isolate(s) can be determined by streaking or patching single colonies onto blood agar plates and observing for a zone of clearance (red blood cell lysis – hemolysis) around the area of growth following overnight (12 to 15 hours) incubation at 37°C. A direct comparison of the level of hemolysis to classical O395 and El Tor N16961 (or C6706) can be made and a score of non-hemolytic (akin to classical O395) or hemolytic (akin to El Tor) can be determined. This result is indicative of whether or not functional hemolysins are produced and secreted by the isolate(s) in question.

Materials

Blood Agar Plates (Fisher Cat. #R01200)

Sterile Wooden Applicator Sticks (Fisher Cat. #01-340))

1. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to include at least the classical wild-type strain O395 and one of either El Tor wild-type strains, N16961 or C6706. Classical O395 is not hemolytic and will therefore not show any clearing around the inoculation point, whereas both N16961 and C6706 are both hemolytic.

2. Holding a wooden applicator stick vertically, touch a single colony with the flat end of the applicator stick.
3. While holding the applicator stick in the same position, “spot” the colony by touching the end of the applicator stick to the surface of the blood agar plate.
 - a. Caution should be made so as not to break through the agar surface.
4. Continue spotting the other strains to be tested as well as the control strains.
 - a. Caution should be taken to space out the inoculating spots on the blood agar plate. Spots should be made with about 2 cm apart to obtain clear hemolysis results.
5. Incubate the plates upside down at 37°C for 48 hrs.

Basic Protocol 6 – Motility Assay

V. cholerae isolates can be characterized as classical or El Tor biotype based on their motility at 37°C. Inoculating the different isolate(s) in question into motility agar and observing the degree of motility compared to classical O395, El Tor N16961 and El Tor C6706 will allow you to determine whether an isolate(s) exhibits decreased motility akin to classical O395 or is highly motile akin to El Tor N16961.

Materials

Motility Agar Plates (see recipe) (Standard size of 100 mm X 15 mm)

Bunsen Burner

Inoculating Wire Stab (Fisher Cat. #22-032-099)

Standard Measuring Ruler

1. Make motility agar plates and allow them to set overnight at room temperature (see Reagents and Solutions for recipe).
2. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to include at least the classical wild-type strain O395 and both El Tor wild-type strains N16961 and C6706.
3. Sterilize the inoculating wire stab using a Bunsen burner (or similar heat source).
4. Using the sterilized inoculating wire stab, touch a single colony and stab the colony through the motility agar straight down vertically to the bottom of the petri dish, and pull straight back up.
 - a. Alternatively, a sterile round toothpick can be used in the same manner as the inoculating wire needle to inoculate the media.
5. Repeat with the other strains. 6. Up to two (2) strains can be inoculated onto one motility plate alongside the three wild-type control strains (Figure 3).

6. Incubate the inoculated plates with the lid up at 37°C for approximately 4 hrs but up to 6 hrs. Do not stack the plates on top of each other.
 - a. The incubation time may vary and growth should be monitored hourly until a clear difference can be observed between the amounts of motile growth.
 - b. El Tor wild-type N16961 will be the most motile control strain, whereas classical O395 is not very motile under these conditions.
7. Measure the diameter of the growth and compare to the wild-type controls.

Basic Protocol 7 – Voges-Proskauer (VP) Assay

The Voges-Proskauer assay detects for the production of acetoin from glucose fermentation, which differs between the classical and El Tor biotypes. After growing cultures of the isolate(s) of interest in MR-VP broth media overnight (12 to 15 hours), the level of acetoin production is determined with the addition of potassium hydroxide and α -naphthol. This protocol describes the preparation of the overnight cultures and the addition of the reagents to visibly determine whether the isolate is VP positive (produces acetoin like wild-type El Tor biotypes) or VP negative (does not produce acetoin as an end-product, like classical O395 biotype).

Materials

MR-VP Broth Media (Difco Cat. #216300) (see recipe)

5% (w/v) α -naphthol (SigmaAldrich Cat. #N1000-10G) (see recipe)

1 M potassium hydroxide (Fisher Cat. #P250-500) (see recipe)

1. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to include at least the classical wild-type strain O395 and both El Tor wild-type strains N16961 and C6706.
2. Make Methyl Red – Voges-Proskauer (MR-VP) broth media (see Reagents and Solutions for recipe).
3. Inoculate 3 ml aliquots of MR-VP broth individually with each strain and incubate overnight at 37°C with aeration.
4. To 1 ml aliquots of each culture, add 130 μ l of 5% (w/v) α -naphthol and 43 μ l of 1 M potassium hydroxide.
5. Vortex briefly and let stand at room temperature for up to 48 hrs to allow full color development.
 - a. Both wild-type El Tor strains N16961 and C6706 are Voges-Proskauer (VP) positive and will develop a clear bright red color throughout the culture.

- b. Wild-type classical O395 is VP negative, but may develop a slight pinkish color after 48 hrs of development.
- c. Strains in question that do not develop any pink color or slight pinkish color after 48 hrs are considered to be VP negative.

Basic Protocol 8 – Biofilm Production Assay

V. cholerae naturally produces biofilm at the liquid-air interface, and the amount of biofilm produced varies not only between classical and El Tor wild-type strains, but even between the El Tor biotype strains N16961 and C6706. This protocol describes how to determine the amount of biofilm that is produced by the isolate(s) of interest and can help in characterization of that isolate(s). Single colonies are required to inoculate the media and it is strongly recommended to include all three wild-type strains, classical O395, El Tor N16961 and El Tor C6706, as controls. For additional information regarding static biofilm assays see Unit 1B.1 (Merritt et al. 2005).

Materials

200 µl 12-channel Multichannel Pipette

0.01% Crystal Violet (see recipe)

50% Glacial Acetic Acid (see recipe)

96-well U-bottom Flexible Plate (BD Cat. #353911) and Lid (BD Cat. #353913)

96-well Flat-bottom Microtiter Plate (Dyner Technologies, Inc. Cat. #3455)

Victor² Multilabel Counter (PerkinElmer Model #1420-018)

1. Streak out the different *V. cholerae* strains for single colonies on a standard rich media agar plate (LB agar) and incubate overnight at 37°C.
 - a. Be sure to include at least the classical wild-type strain O395 and both El Tor wild-type strains N16961 and C6706.
2. Grow overnight cultures of each strain in LB at 30°C.
3. Make 1:100 dilutions of each culture and aliquot 100 µl of into at least 3 wells of a 96-well plate.
 - a. Aliquoting into 3 wells for each strain will allow for a technical triplicate. However, it is common to aliquot the same culture into 8 wells (one column of a 96-well plate) for convenience when taking pictures (step 12) and the added increase in replicates.
 - b. Be sure to reserve wells for the three control wild-type control strains as well as an equal number of wells for a sterile culture media control.
4. Incubate the 96-well plate at the desired temperature (generally 30 or 37°C) for 24 hrs.

5. Dump the contents of the plate into a waste container and wash the wells two times with the same media used to grow the cultures. The biofilm will form on the sides of the wells at the liquid-air interface, and therefore you will not lose any significant amounts of the biofilm when the plate is inverted and the samples dumped out.
 - a. Cross contamination at this point will not affect results as the amount of biofilm production measured requires growth.
 - b. Follow disposal regulations according to your institution's environmental health and safety office.
6. Tap the plate upside down onto a paper towel to remove as much residual media as possible.
7. Add 200 μ l of 0.01% (w/v) crystal violet to each well using a multichannel pipettor, and incubate the plate for 5 minutes at room temperature.
 - a. The crystal violet will irreversibly bind to the polysaccharide structure of the biofilm.
8. Dump the crystal violet into a waste beaker and dispose of accordingly.
 - a. Follow disposal regulations according to your institution's environmental health and safety office.
9. Wash the plate under running water, while shaking the water out of the wells repeatedly.
 - a. Extensive washing to remove any unbound excess crystal violet is recommended for accurate results, and thus may require up to ten or more rinse and shake cycles.
10. Shake out the excess water and tap the plate upside down onto paper towels to remove as much residual water as possible.
11. Allow the plate to air dry before continuing on to the next step.
12. At this point, a representative set of wells (one row) may be cut out and a picture taken to show the amount of biofilm produced by the different strains and stained with the crystal violet.
13. To the remainder of the plate, add 150 μ l of 50% (v/v) glacial acetic acid to all the wells using a multichannel pipettor.
14. Dissolve the crystal violet in the wells by repeatedly pipetting the glacial acetic acid up and down.
15. Transfer the contents of the wells to a new flat bottom 96-well microtiter plate and read the plate at 550 nm.
16. On a bar graph, graph the OD₅₅₀ along the y-axis against the different strains along the x-axis.

Reagents and Solutions

1.25 mM dNTP Mix

100 mM dATP – 12.5 μ l (Final Concentration 1.25 mM)

100 mM dTTP – 12.5 μ l (Final Concentration 1.25 mM)

100 mM dCTP – 12.5 μ l (Final Concentration 1.25 mM)

100 mM dGTP – 12.5 μ l (Final Concentration 1.25 mM)

Sterile double-distilled water – up to 1000 μ l

1% Agarose Gel

UltraPure Agarose – 1.0 g

1X TBE – 100 ml

1X TBE (Tris-borate-EDTA) Buffer (1000 ml)

Tris base – 5.4 g (Final Concentration 44.58 mM)

Boric acid – 2.75 g (Final Concentration 44.48 mM)

0.5 M EDTA (pH 8.0) – 2.0 ml (Final Concentration 0.1 mM)

Sterile double-distilled water – up to 1000 ml

6X DNA Gel Loading Dye (20 ml)

Bromophenol Blue – 0.05 g (Final Concentration 0.075 mM)

100% Glycerol – 6.0 ml (Final Concentration 30% v/v)

0.5 M EDTA (pH 8.0) – 40.0 μ l (Final Concentration 1 mM)

1 M Tris-HCl (pH 8.0) – 1.0 ml (Final Concentration 50 mM)

90X Gel Green – 2.0 ml (Final Concentration 9X) (Biotium Cat. #41005-0.5ml)

Sterile double-distilled water – up to 20.0 ml

Minimal Citrate Media Agar Plates

*Autoclave the sterile water and agar first.

Agar – 3.0 g (BD Cat. #214030)

Sterile double-distilled water – 180 ml

Autoclave, and then add:

10X VBMM – 20 ml (Final Concentration 1X)

10X VBMM (for Minimal Citrate Media Plates)

MgSO₄•7H₂O – 0.2 g

Citric Acid•H₂O – 2.0 g

K_2HPO_4 (anhydrous) – 10.0 g

$\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ – 3.5 g

Double-distilled water – up to 100 ml

This can be autoclaved and stored at room temperature indefinitely.

Milk Agar Plates

*Components must be prepared separately and mixed after autoclaving

*After mixing, allow media to cool prior to pouring of plates

Component 1

Instant Non-fat Dry Milk – 4.0 g (Nestle Carnation)

Double-distilled water – up to 100 ml

Component 2

Brain-Heart Infusion – 1.84 g (Difco Cat. #237500)

Agar – 3.0 g (BD Cat. #214030)

Double-distilled water – up to 100 ml

Motility Agar Plates

*These plates must be poured thicker (40 ml/plate) than normal agar plates (20 ml/plate)

Tryptone – 5.0 g (Final Concentration 1% (w/v))

Yeast Extract – 2.5 g (Final Concentration 0.5% (w/v))

NaCl – 2.5 g (Final Concentration 0.5% (w/v))

Agar – 1.5 g (Final Concentration 0.3% (w/v))

Double-distilled water – up to 500 ml

Voges-Proskauer Broth and Reagents

MR-VP Broth

MR-VP Media – 1.7 g

Sterile double-distilled water – up to 100 ml

5% (w/v) α -naphthol

α -naphthol

α – 1 g (Final Concentration 5% (w/v))

Sterile double-distilled water – up to 20.0 ml

1 M potassium hydroxide

Potassium Hydroxide – 2.8 g (Final Concentration 1 M)

Sterile double-distilled water – up to 50.0 ml

Biofilm Assay Reagents

0.01% (w/v) Crystal Violet (Fisher Cat. #C-581)

1% (w/v) Crystal Violet – 0.5 ml (Final Concentration 0.01% (w/v))

Sterile double-distilled water – up to 50.0 ml

50% (v/v) Glacial Acetic Acid (Fisher Cat. #A38S-212)

Glacial Acetic Acid – 10.0 ml (Final Concentration 50% (v/v))

Sterile double-distilled water – 10.0 ml

Commentary

Background Information

Vibrio cholerae is a gram-negative aquatic bacterium that is the causative agent of the secretory diarrheal disease cholera. Although over 200 serogroups have been identified based on the structure of the cell surface lipopolysaccharide O-antigen, historically, only 2 of these serogroups, O1 and O139, have been known to cause or have the potential to cause cholera epidemics. Serogroup O139 caused a relatively brief pandemic from 1992 to 1995 and has since not been linked to any outbreaks. The clinically more relevant serogroup, O1, is further sub-divided into the 2 biotypes classical and El Tor, with independently evolved lineages (Kaper et al. 1982; Karaolis et al. 1995) resulting in genotypic and phenotypic differences. While the more toxic O1 classical biotype was responsible for the first six pandemics, it has since been displaced by the more persistent O1 El Tor biotype (Sack et al. 2004), which is the causative agent of the ongoing seventh pandemic. Interestingly, the recently identified responsible agent in the 2010 cholera outbreak in Haiti, resulting in more than 231,000 reported cases and 4,500 deaths over the first five months of the outbreak (2011), has been shown to be of the El Tor biotype (Chin et al. 2011).

Reports dating from 2001 have documented El Tor biotype strains isolated at various times since the early 1990's at Matlab Hospital in Bangladesh and more recently from other parts of Asia (Nair et al. 2002; Nair et al. 2006a; Nguyen et al. 2009), Mozambique (Lee et al. 2006) and Haiti (Chin et al. 2011), which possess some classical genotypic and phenotypic traits. These El Tor isolates have been dubbed El Tor variants and have added a new complexity to the once simple identification process employed previously. Currently, isolated clinical and/or environmental strains must be characterized based on a series of genetic and phenotypic assays in order to characterize the isolates beyond just the El Tor background. Although other assays are available, such as definitive genetic screens (deep sequencing), they are largely unnecessary and may be very costly, especially considering the purpose of conducting such large-scale and labor intensive experiments. The set of protocols described in this unit provides simple genetic and phenotypic screens that can be quickly performed at a relatively low cost, yet provide the necessary results to help identify and initiate characterization of clinical and/or environmental *V. cholerae* isolates.

Critical Parameters and Troubleshooting

Vibrio cholerae is a biosafety level 2 (BSL 2) organism that is the causative agent of the potentially fatal diarrheal disease cholera. As with handling any BSL 2 organism, proper training and handling should be in place and waste materials be properly handled and disposed of according to individual institutional guidelines.

Because of the extra value placed on clinical and/or environmental isolates, samples should be handled carefully so as to prevent any cross-contamination and that stock cultures of each isolate be properly made and maintained. For proper storage and maintenance of *V. cholerae* strains, please refer to (Martinez et al. 2010).

For genetic screens of the *ctxB* and *tcpA* genes, care should be taken when designing primers to maximize expected PCR products and reduce non-specific bands, as with any PCR. Inoculation on the different media plates for phenotypic assays should be conducted carefully on plates poured the day prior to inoculation. This will allow sufficient “drying” of the plates and remove any concerns of liquid on the surface of the plates prior to inoculation. For the polymyxin B resistance assay, the polymyxin B should be added to the cooled molten agar after autoclaving to prevent destruction of the antibiotic. HapR milk agar plate inoculation should be performed so as to allow a large enough space between inoculation streaks (or patches) so that zones of clearance do not overlap with each other. This can generally be avoided by limiting the size of the streak (or patch) to the suggested numbers as outlined in Basic Protocol 4. Similarly, as hemolysis will also be visually inspected for a zone of clearance, ample spacing should also be accommodated when inoculating the blood agar plates. Motility assays should be continuously monitored over the course of the assay (approximately every hour). This will allow the user to stop and make observations as maximal motility has been achieved without the colonies growing into one another.

Anticipated Results

As this set of protocols details the characterization of clinical and/or environmental isolates, it is impossible to predict what the results will be for any given strain. Therefore the importance of running positive and negative control strains in the form of the wild-type classical O395 and wild-type El Tor N16961 and C6706, whenever possible cannot be over-emphasized. The inclusion of all three wild-type strains will allow for easier interpretation of the data generated by each of the assays, allowing characterization of the isolates more reliable.

Time Considerations

The genetic screen (sequencing of *tcpA* and *ctxB*) will require one day to obtain single colonies, followed by growth of an overnight culture (12 to 15 hours). Isolation of the DNA using standard commercially available kits should take about 30 to 60 min depending on the number of samples. A break in the protocol can be taken here and the samples stored up to 1 week at 4°C. Verification on a 1% agarose gel should take about 2 hours at 150 volts, and purification of the PCR products using commercially available kits should take another 30 min before samples are ready to be sequenced. Purified PCR samples can be stored up to 1

week at 4°C or stored indefinitely at –20°C, and thawed when ready to be sequenced. Consult your local sequencing facility for turnaround times for PCR sequencing.

Polymyxin B resistance, citrate metabolism, casein hydrolysis protease activity and hemolysis assays can all be accomplished simultaneously using the different media plates. Inoculation on each media requires only a few minutes per sample and observations can be made the following day after 12 to 15 hours of incubation. The plates can then be stored up to 3 days at 4°C if observations cannot be made immediately.

The motility assay can take up to 6 hours after inoculation, with intermittent monitoring every hour, and so ample time should be allotted to make observations, including final measurements of the diameter of growth.

The Voges-Proskauer (VP) assay requires approximately 48 hours from the time of inoculation to the time of final observation, but necessitates very limited handling or processing time. After the addition of the potassium hydroxide and α -naphthol to the overnight culture, the samples can be left at room temperature for 48 to 72 hours. However it is recommended to make observations around 48 hours for maximum effects.

The biofilm assay is completed over a course of three days, with minimal handling of the samples in-between. Each day will require approximately 1 hour for processing, with the final day requiring up to 2 hours for processing, depending on the total number of samples.

All assays can be run simultaneously as the times for observations will vary and do not require much time. However, for someone that is characterizing isolates for the first time and who is not familiar with these techniques, it is recommended to perform the assays individually or in groups. For example, the motility assay can be started and left incubating while the genetic screen is set-up, the PCR run, and the samples run on the agarose gel. On the following day, all the media plates can be inoculated and cultures started for the VP and biofilm assays.

Acknowledgments

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		1	2	3	4	5		
	6	7	8	9	10	11	12	
13	14	15	16	17	18	19	20	21
22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39
40	41	42	43	44	45	46	47	48
49	50	51	52	53	54	55	56	57
	58	59	60	61	62	63	64	
		65	66	67	68	WT		

Figure 1.

Sample grid used for patching. This grid can be used for testing citrate metabolism, where single colonies are patched individually into each numbered box. Notice only one box is marked WT for a wild-type control, however, other boxes may be used in the case of multiple controls, both positive and negative.

Figure 2.
Milk agar plate used for casein hydrolysis by protease activity testing of classical O395 (HapR negative), El Tor C6706 (HapR positive), El Tor N16961 (HapR negative) and three El Tor variants (Var 1, Var 2 and Var 3 – all HapR positive).

Figure 3.

Sample motility assay showing the relatively non-motile nature of classical O395, motile El Tor C6706, very motile N16961, and two variants (Iso #1 and Iso #2) with motility patterns similar to that of El Tor N16961.

1 μ l	chromosomal DNA (100 – 200 ng)
8 μ l	1.25 mM dNTP mix
5 μ l	10X Pfu buffer
1 μ l	Forward primer (30 pmole/ μ l)
1 μ l	Reverse primer (30 pmole/ μ l)
1 μ l	Pfu (2.5 Units/ μ l)
up to 50 μ l	Sterile double-distilled water

Table 6A.2.1Primers Used for PCR Amplification and Sequencing of *tcpA* and *ctxB*

Primer name	Sequence (5'→3')	PCR product size (bp)
<i>tcpA</i> -For	CCGCACCAGATCCACGTAGGTGGG	1420
<i>tcpA</i> -Rev	GTCGGTACATCACCTGCTGTGGGGCAG	1420
<i>ctxB</i> -For	GGGAATGCTCCAAGATCATCGATGAGTAAT AC	580
<i>ctxAB</i> -Rev	CATCATCGAACCACAAAAAGCTTACTGAG G	580