Video Article

Laboratory Techniques Used to Maintain and Differentiate Biotypes of *Vibrio cholerae* Clinical and Environmental Isolates

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Abstract

The aquatic Gram-negative bacterium *Vibrio cholerae* is the etiological agent of the infectious gastrointestinal disease cholera. Due to the global prevalence and severity of this disease, *V. cholerae* has been extensively studied in both environmental and laboratory settings, requiring proper maintenance and culturing techniques. Classical and El Tor are two main biotypes that compose the *V. cholerae* O1 serogroup, each displaying unique genotypic and phenotypic characteristics that provide reliable mechanisms for biotype characterization, and require distinct virulence inducing culturing conditions. Regardless of the biotype of the causative strain for any given infection or outbreak, the standard treatment for the disease involves rehydration therapy supplemented with a regimen of antibiotics. However, biotype classification may be necessary for laboratory studies and may have broader impacts in the biomedical field. In the early 2000's clinical isolates were identified which exhibit genotypic and phenotypic traits from both classical and El Tor biotypes. The newly identified hybrids, termed El Tor variants, have caused clinical and environmental isolate biotype identification to become more complex than previous traditional single assay identification protocols. In addition to describing *V. cholerae* general maintenance and culturing techniques, this manuscript describes a series of gene specific (*ctxB* and *tcpA*) PCR-based genetic screens and phenotypic assays (polymyxin B resistance, citrate metabolism, proteolytic activity, hemolytic activity, motility, and glucose metabolism via Voges-Proskauer assay) collectively used to characterize and/or distinguish between classical and El Tor biotypes. Together, these assays provide an efficient systematic approach to be used as an alternative, or in addition, to costly, labor-intensive experiments in the characterization of *V. cholerae* clinical (and environmental) isolates.

Video Link

The video component of this article can be found at https://www.jove.com/video/55760/

Introduction

Cholera is a disease of the distal small intestine caused by the consumption of contaminated food or water containing the aquatic Gram-negative bacterium *Vibrio cholerae*. Symptoms of cholera include vomiting and uncontrollable watery diarrhea, leading to severe dehydration, which if not treated properly, will result in death. *V. cholerae* can be divided into over 200 serogroups based on the structure of the cell-surface lipopolysaccharide O-antigen. However, only 2 serogroups, O1 and O139, have shown epidemic or pandemic potential[1,2]. Moreover, serogroup O139 has been primarily isolated to Southeastern Asia[3,4], while serogroup O1 is distributed worldwide. Furthermore, the O1 serogroup can be divided into 2 main biotypes: classical and El Tor. The classical biotype was responsible for the first 6 cholera pandemics between 1817 and 1923. The ongoing seventh pandemic is a result of the El Tor biotype, which has globally displaced the classical biotype in the environment[5,6,7]. Recently, strains have arisen which contain distinguishing characteristics of both classical and El Tor biotypes[8,9,10,11,12,13,14,15,16,17]and have since been termed El Tor variants[13,17]. Some El Tor variants have demonstrated elevated virulence capabilities with more rapid and severe disease progression than previously observed, emphasizing the need for a more comprehensive approach to agent identification and disease prevention and treatment[8,9,18]. While biotype identification does not immediately dictate treatment, further advancements in vaccine development and future therapeutic agents may benefit from biotype distinction.

The first series of protocols listed here will enable investigators to properly maintain *V. cholerae* strains in a laboratory setting. Consistency and subsequent analysis requires stock preparation and growth of isolates, which is not biotype-dependent. However, to optimally induce virulence gene expression, independent biotype specific culturing techniques are required[19]. Additionally, preparation for various genetic and biochemical assays are outlined in this manuscript.

Cholera toxin (CT) and the toxin co-regulated pilus (TCP) are two main virulence factors controlled by the master regulator ToxT in both biotypes of the *V. cholerae* O1 serogroup[20]. CT is a bipartite toxin composed of five CtxB subunits surrounding a single CtxA subunit, and is responsible for the rapid electrolyte loss associated with cholera. TCP is a type IV pilus encoded by the *tcp* operon (*tcpABQCRDSTEF*), and is involved in attachment and colonization of the distal small intestine. *tcpA* is the first gene of the *tcp* operon which encodes the individual pilin subunits essential for construction of the pilus[8]. The gene sequence for *ctxA* is completely conserved between classical and El Tor biotypes, while *ctxB*

and *tcpA* differ across the two biotypes but are conserved within each biotype[8]. *ctxB* is completely conserved between biotypes except at two base positions (115 and 203). In the El Tor biotype, thymine resides at base positions 115 and 203, while the classical biotype contains cytosine at these bases. *tcpA* is completely conserved within each biotype, yet differ at multiple bases between biotypes. These genetic distinctions serve as primary biotype identification markers, and after sequencing the polymerase chain reaction (PCR) amplification product including these sites, isolate sequences can be compared to wild-type (WT) classical O395 or WT El Tor N16961 to determine the biotype background of CT and TCP, respectively, in a given *V. cholerae* isolate.

Numerous protocols have been developed to characterize the phenotypic distinctions between the classical and El Tor biotypes[21,22,23]. Polymyxin B is a peptide antibiotic that compromises the integrity of the outer cell membrane in Gram-negative bacteria, and polymyxin B resistance can be visualized through the polymyxin B resistance assay[21]. Citrate is a primary substrate of the Kreb's cycle, and the ability to metabolize citrate as a sole carbon source can be determined using the citrate metabolism assay[22]. *hapR* encodes a global regulator and the master quorum-sensing regulator in *V. cholerae,* HapR, which binds to various promoter regions and regulates gene and operon expression[24]. Some pathogenic strains of *V. cholerae* have a naturally occurring frame-shift mutation in the *hapR* gene that has caused this density dependent regulation of virulence gene expression to be lost[24,25]. Measuring HapR-regulated protease activity using milk agar media allows the researcher to identify whether a particular isolate contains a functional HapR[23]. The hemolysis assay tests for a strain's ability to secrete hemolytic enzymes that lyse red blood cells; the degree of hemolysis can be visualized on blood agar plates[23]. Motility is often associated with virulence in *V. cholerae* and can be analyzed using motility agar plates[23]. The Voges-Proskauer assay tests for a strain's ability to ferment glucose as a sole carbon source and produce the byproduct acetoin[21]. With the emergence of El Tor variants, it is difficult to predict the results of any given phenotypic assay without extensive genotypic screening, and before deducing the biotype background of *V. cholerae* isolates, it is recommended to perform this assembly of assays[23] and compare the results to reference strains as in **Table 2**.

Herein, we have advanced a series of protocols, collectively utilizing the aforementioned genotypic and phenotypic assays for a more comprehensive approach to characterizing *V. cholerae* biotypes. Furthermore, we have described the genotypic and phenotypic distinctions of known *V. cholerae* El Tor variants (MQ1795 and BAA-2163), in comparison to commonly used biotype reference strains (WT classical O395, WT El Tor C6706, and WT El Tor N16961; **Table 1**). The emergence of El Tor variants has presented challenges to the reliability of previously employed single assay biotype characterization protocols; however, this multiple assay identification system will allow for more reliable characterization of clinical and environmental *V. cholerae* isolates.

Protocol

Note: Time considerations for each assay must be made as individual media preparations require different times. For example, solid agar plate media should be allowed to sufficiently cool and dry (1-2 days). Additional time considerations (*i.e.* single colony and overnight culture growth) are specified under each protocol and are found in **Table 2**.

1. Preparation of Media

1. **1x Phosphate Buffered Saline (PBS)**
    1. Weigh 4.0 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, and 0.12 g KH_2PO_4. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 500 mL with deionized water, adjust pH to 7.2 using a pH meter and adding HCl dropwise to the solution, and autoclave or filter sterilize using a 0.22 µm filter. 1x PBS can be stored at room temperature indefinitely.
    CAUTION: HCl is a concentrated acid and should be handled according to institutional, local, state, and federal regulations. For appropriate handling guidelines refer to the safety data sheet provided by the manufacturer.

2. **Luria-Bertani (LB) Broth**
    1. Weigh 5.0 g tryptone, 2.5 g yeast extract, and 2.5 g NaCl. Combine the constituents in a 1 L bottle, adjust volume to 500 mL with deionized water, autoclave, and store at room temperature for up to 6 months. For classical biotype virulence inducing conditions, adjust pH to 6.5 using HCl prior to autoclaving.

3. **AKI Medium Containing 0.03% (w/v) $NaHCO_3$**
    1. For Component 1 (AKI medium): weigh 7.5 g peptone, 2.0 g yeast extract, and 2.5 g NaCl. Combine the constituents in a 1 L bottle, adjust volume to 450 mL with deionized water and autoclave. AKI medium can be stored at room temperature for up to 6 months.
    2. For Component 2 ($NaHCO_3$): weigh 1.5 g $NaHCO_3$, and adjust volume to 50 mL with deionized water in a sterile vesicle. Filter sterilize $NaHCO_3$ using a 0.22 µm filter. $NaHCO_3$ must be prepared immediately before use.
    3. Aseptically combine the two components creating a 1:10 ratio by filtering Component 2 into Component 1 prior to use.

4. **Luria-Bertani (LB) Agar Plates**
    1. Weigh 5.0 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, and 7.5 g agar. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 500 mL with deionized water, autoclave, and prepare in standard sized 100 mm x 15 mm sterile Petri dishes. Plated media can be stored wrapped in plastic for up to 6 months, lid-side up at 4 °C.

5. **LB Agar Plates Supplemented with Polymyxin B**
    1. For Component 1 (LB agar): weigh 5.0 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, and 7.5 g agar. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 500 mL with deionized water and autoclave.
    2. For Component 2 (polymyxin B): dissolve polymyxin B in deionized water in a sterile 2 mL microcentrifuge tube to a concentration of 50,000 IU/µL and filter sterilize using a 0.22 µm filter.
    3. Once Component 1 is cool to the touch, but not yet solidified, aseptically add 500 µL of Component 2 to Component 1 creating a final concentration of 50 IU/µL and mix thoroughly. Prepare in standard sized 100 mm x 15 mm sterile Petri dishes by pouring mixed agar media into the Petri dishes. Plated media can be stored wrapped in plastic for up to 3 months, lid-side up at 4 °C.

6. **Minimal Citrate Medium Agar Plates**
    1. For Component 1 (agar with bromothymol blue): weigh 7.5 g agar and 0.02 g bromothymol blue. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 450 mL with deionized water and autoclave the mixture.
    2. For Component 2 (10x VBMM): weigh 1.0 g $MgSO_4 7H_2O$, 10.0 g citric acidH_2O, 50.0 g anhydrous K_2HPO_4, and 17.5 g $NaNH_4HPO_4 4H_2O$. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 500 mL with deionized water and autoclave or filter sterilize using a 0.22 µm filter. 10x VBMM can be stored at room temperature up to 6 months.
    3. Add 50 mL of Component 2 to Component 1 and prepare in standard sized 100 mm x 15 mm sterile Petri dishes by pouring mixed agar media into the Petri dishes. Plated media can be stored wrapped in plastic for up to 6 months, lid-side up at 4 °C.

7. **Milk Agar Plates**
    1. For Component 1 (milk): weigh 8.0 g instant nonfat dry milk in a 1 L Erlenmeyer flask, adjust volume to 200 mL with deionized water and autoclave.
    2. For Component 2 (agar with brain-heart infusion): weigh 3.68 g brain-heart infusion and 6.0 g agar. Combine the constituents in a 500 mL Erlenmeyer flask, adjust volume to 200 mL with deionized water and autoclave.
    3. Combine the two components by pouring Component 2 into Component 1, after autoclaving and mix thoroughly. Prepare in 150 mm x 15 mm sterile Petri dishes. Milk plates can be stored for up to one week wrapped in plastic, lid-side down at 4 °C. Prepare in 150 mm x 15 mm sterile Petri dishes by pouring mixed agar media into the Petri dishes.

8. **Motility Agar Plates**
    1. For Component 1 (LB agar): weigh 5.0 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, and 2.0 g agar. Combine the constituents in a 1 L Erlenmeyer flask, adjust volume to 500 mL with deionized water and autoclave.
    2. For Component 2 (1% (w/v) triphenyltetrazolium chloride; TTC): weigh 0.25 g TTC. Adjust volume to 25 mL with deionized water in a sterile vesicle and filter sterilize using a 0.22 µm filter. Store for up to 6 months at room temperature wrapped in foil to minimize light exposure.
    3. Add 2.5 mL of 1% (w/v) sterile TTC to autoclaved media.
    4. Pour media as thick as possible (≥50 mL/plate) into large 150 mm x 15 mm sterile Petri dishes. Plated media can be stored wrapped in plastic for up to one week, lid-side up at 4 °C. Prepare in 150 mm x 15 mm sterile Petri dishes by pouring mixed agar media into the Petri dishes.

9. **Voges-Proskauer (VP)**
    1. For Component 1 (Methyl Red–Voges-Proskauer broth; MR–VP): weigh 1.7 g MR-VP medium in a 500 mL Erlenmeyer flask, adjust volume to 100 mL with deionized water and autoclave. Sterile MR-VP broth can be stored at room temperature for up to 6 months.
    2. For Component 2 (5% (w/v) alpha (α) naphthol): weigh 1.0 g α-naphthol in a sterile vesicle and adjust volume to 20 mL with 95% ethanol. α-naphthol can be stored at room temperature for up to 1 week wrapped in foil to minimize light exposure.
    3. For Component 3 (40% (w/v) potassium hydroxide; KOH): weigh 20.0 g KOH in a sterile vesicle, adjust volume to 50 mL with sterile deionized water. KOH can be stored for up to 6 months at room temperature in a plastic vessel.

2. Maintenance and Growth of *V. cholerae* Strains

1. **Preparation and Use of Frozen Stock Cultures**
    1. Pellet 1.8 mL of liquid overnight culture for 2 min by centrifugation (≥8,600 x g) in a sterile 2 mL microcentrifuge tube, remove supernatant, and re-suspend the cell pellet in 900 µL of fresh LB broth by pipetting.
    2. Add 900 µL of sterile 60% (v/v) glycerol to the culture and mix by vortexing.
    3. Transfer the mixture to a sterile 2 mL cryogenic tube and store indefinitely at -80 °C.
    4. To use, remove a small amount of frozen stock using a sterile inoculating loop and streak for single colonies on LB agar plates. Immediately return frozen stock to -80 °C after use to prevent cultures from completely thawing. Incubate plates lid-side down for 12 to 16 h at 37 °C.

2. **Growth of Overnight Cultures in Liquid Medium**
    1. Streak for single colonies (as per section 2.1.4) from frozen stock onto LB agar plates. Incubate plates lid-side down for 12 to 16 h at 37 °C.
    2. Inoculate 4 mL of liquid LB broth in a sterile 10 mL culture tube with a single colony by touching the surface of the single colony with a sterile applicator stick and transferring the colony into the liquid broth.
    3. Incubate with aeration in a shaker incubator at 225 rpm for 12 to 16 h at 37 °C.

3. **Growth Curve**
    1. Prepare overnight culture in liquid LB broth as previously stated in protocol 2.2.
    2. Make a 1:100 dilution of overnight culture by transferring 250 µL of overnight culture to 25 mL of fresh LB broth in a sterile 250 mL Erlenmeyer flask.
    3. Measure the optical density of the liquid cultures at 600 nm (OD_{600}) every hour beginning at the time of inoculation (T_0).
    4. Incubate a 1:100 dilution of overnight culture with aeration in a shaker incubator at 225 rpm for up to 30 h at 37 °C, or until culture reaches maximal density.
    5. Graph the OD_{600} vs. Time and use linear regression to determine the approximate doubling time for each strain.

4. **El Tor Biotype Virulence Inducing Conditions**
    1. Streak for single colonies from frozen stock onto LB agar plates. Incubate plates lid-side down, for 12 to 16 h at 37 °C.
    2. Inoculate 10 mL of AKI medium containing 0.03% (w/v) $NaHCO_3$ with a single colony.
    3. Incubate culture without aeration at 37 °C for 3.5 h.

4. Remove 7 mL of culture and incubate the remaining 3 mL of culture with aeration in a shaker incubator at 225 rpm for an additional 4 h.

5. **Classical Biotype Virulence Inducing Conditions**
    1. Streak for single colonies from frozen stock onto LB agar plates. Incubate plates lid-side down for 12 to 16 h at 37 °C.
    2. Inoculate 4 mL liquid LB broth (pH 6.5) with a single colony.
    3. Incubate with aeration in a shaker incubator at 225 rpm for 12 to 16 h at 30 °C.

6. **Washing Cell Pellet in 1x PBS**
    1. Pellet 1.8 mL of overnight culture for 2 min by centrifugation (≥8,600 x g) in a sterile 2 mL microcentrifuge tube and remove the supernatant using a pipette. Re-suspend cell pellet in 1.8 mL 1x PBS by pipetting.
    2. Repeat washing procedure three times followed by a final resuspension in 1.8 mL 1x PBS.

3. Characterizing *V. cholerae* Biotypes

1. **PCR-based Genetic Screens using *ctxB* and *tcpA***
    1. Design a set of primers, which anneal approximately 50-70 bp upstream and downstream of the translational start and stop sites of *ctxB* and *tcpA*, respectively.
    2. Prepare overnight culture in liquid LB broth as previously stated in protocol 2.2.
    3. Isolate chromosomal DNA using a commercially available kit designed for Gram-negative bacteria. Purified chromosomal DNA can be stored indefinitely at -20 °C. Chromosomal DNA isolation using commercially available kits generally take about 4 h.
    4. Use a micro-volume spectrophotometer to ensure the chromosomal DNA sample is of high quality ($A_{260/280}$ >1.8).
    5. For each chromosomal DNA isolate, prepare polymerase chain reaction(s) (PCR) on ice in sterile 200 µL PCR tube(s) and amplify regions of *ctxB* and *tcpA* using standard PCR components: *Taq* polymerase and buffer (or equivalents), dNTP solution, and forward/reverse primers. Use standard PCR parameters (~3 h). For example, use the following protocol:
        1. Initial denature at 95 °C for 120 s.
        2. Denature at 95 °C for 60 s.
        3. Anneal primers at 60 °C for 45 s.
        4. Extend at 72 °C for 90 s.
        5. Repeat steps 3.1.5.2 through 3.1.5.4 for 34 cycles.
        6. Final extension 72 °C for 600 s.
        7. Infinite hold at 4 °C.
    6. Dye 5 µL of respective PCR product(s) using a standard 6x DNA gel loading dye, and load approximately 10 µL of 1 kb ladder and 10 µL of PCR product onto a 1% agarose gel in 1x Tris-Borate-EDTA (TBE) buffer.
    7. Run gel electrophoresis at 130 V until dye front reaches the end, but not off, of the gel (approximately 90 min). Constant monitoring is recommended as voltage and running times can vary depending on the equipment.
    8. Upon verification of successful PCR amplification at the expected size, purify the remaining 45 µL PCR product(s) using a commercially available DNA clean & concentrator kit.
    9. Sequence cleaned PCR product(s) using the same primers used for PCR amplification, and prepare per local sequencing facility guidelines.
    10. Compare the FASTA format of gene sequences from each isolate to the published sequence available on the NCBI website (http://www.ncbi.nlm.nih.gov/gene/), by searching the following accession numbers in the search query box.
    11. *ctxB*: (classical) region between VC0395_A1059 to VC0395_A1060 (El Tor) VC_1456
    12. *tcpA*: (classical) VC0395_A0353 (El Tor) VC_0828

2. **Phenotypic Assays for Biotype Classification via Spotting**
    1. Prepare overnight culture in liquid LB broth as previously stated in protocol 2.2.
    2. Wash the cell pellet(s) as specified in protocol 2.6.
    3. Use a pipette to spot 1 µL of washed culture on respective medium. For medium selection and incubation specifications, refer to Table 2.

3. **Motility Assay**
    1. Prepare overnight culture(s) in liquid LB broth as previously stated in protocol 2.2.
    2. Wash the cell pellet(s) as specified in protocol 2.6.
    3. Inoculate motility agar plates by inserting inoculating stab into the washed liquid culture and "stab" vertically into the media. In between each inoculation, sterilize the wire stab using a Bunsen burner, and when stabbing agar, ensure the inoculating stab does not bend, as this can alter results.
    4. Incubate plates lid-side up for 14 to 24 h at 37 °C. After 14 h, monitor motility plates closely to prevent overgrowth of cultures.

4. **Voges-Proskauer (VP) Assay**
    1. Prepare overnight culture(s) in liquid LB broth as previously stated in protocol 2.2.
    2. Inoculate 4 mL of methyl red Voges-Proskauer, or MR-VP broth, by pipetting 10 µL of previously prepared overnight culture into 4 mL MR-VP broth in a sterile culture tube and incubate with aeration in a shaker incubator at 225 rpm for 12 to 16 h at 37 °C.
    3. Add 150 µL of 5% (w/v) α-naphthol and 50 µL 40% (w/v) KOH to 1 mL aliquots of MR-VP overnight culture in sterile culture tubes, respectively.
    4. Briefly vortex and let stand at room temperature for up to 4 h until color change develops.

Representative Results

For proper maintenance and use of any bacterial strain, it is recommended to know the doubling time of the strain(s) of interest. Herein, the varying growth rates of commonly used *V. cholerae* strains were demonstrated through a growth curve, and approximate doubling times were calculated using linear regression. WT El Tor N16961 and El Tor variant MQ1795 demonstrated shorter doubling times (~1 h and ~1 h, respectively) than WT classical O395 (~2 h) (**Figure 1**; **Table 2**).

V. cholerae genetic manipulation and subsequent analysis often relies on the ability to properly distinguish between biotypes. PCR based genetic screens and phenotypic assays were collectively implemented as a reliable system for distinguishing between biotype backgrounds of *V. cholerae* clinical and environmental isolates; for representation, biotype reference strains (WT classical O395, WT El Tor C6706, and WT El Tor N16961) and representative El Tor variants (MQ1795 and BAA-2163) were included (**Table 1**). WT classical O395 demonstrated classical *ctxB* and *tcpA* sequences. Conversely, WT El Tor strains N16961 and C6706 demonstrated El Tor *ctxB* and *tcpA* sequences. Interestingly, MQ1795 and BAA-2163 contained the classical biotype *ctxB* subunit comparable to O395, yet both El Tor variants contained the *tcpA* indicative of the El Tor biotype background (**Table 1**). WT classical biotype strain O395 showed sensitivity to polymyxin B, while WT El Tor biotype strains (C6706 and N16961) showed resistance and exhibited growth on LB agar plates supplemented with polymyxin B. The representative El Tor variant strains (MQ1795 and BAA-2163) demonstrated similar resistance to the antibiotic relative to the WT El Tor biotype strains (C6706 and N16961) (**Figure 2**; **Table 2**). WT classical biotype strain O395 did not grow on minimal citrate media, while WT El Tor strains (C6706 and N16961) were able to utilize citrate as a carbon source and exhibit growth on minimal citrate media. Representative El Tor variant biotype strains (MQ1795 and BAA-2163) demonstrated growth comparable to that of the WT El Tor biotype strains (C6706 and N16961) (**Figure 3**; **Table 2**). WT classical strain O395 and WT El Tor strain N16961 possess a non-functional HapR, and, thus did not demonstrate HapR-regulated protease activity; WT El Tor strain C6706, and representative El Tor variants (MQ1795 and BAA-2163) are *hapR*-positive-visualized as a zone of clearance emanating from the point of inoculation (**Figure 4**; **Table 2**). WT classical strain O395 does not secrete hemolytic enzymes and was therefore γ-hemolytic, while WT El Tor biotype strains (C6706 and N16961) and representative El Tor variant strains (MQ1795 and BAA-2163) secrete hemolytic enzymes that completely lyse red blood cells surrounding the point of inoculation and showed β-hemolysis (**Figure 5**; **Table 2**). Motility varies across, and within, biotype strains; however, WT El Tor strain N16961 and El Tor variants (MQ1795 and BAA-2163) demonstrated hyper-motility when compared to the relatively less motile WT classical strain O395 and WT El Tor strain C6706 (**Figure 6**; **Table 2**). WT classical strain O395 and representative El Tor variants did not metabolize glucose to produce acetoin, while WT El Tor biotype strains produced acetoin as a byproduct of glucose fermentation, as indicated by development of a deep red color during the Voges-Proskauer assay (**Figure 7**; **Table 2**).

Strain	***ctxB* Gene**			***tcpA***	**Reference**
	Base 115	**Base 203**	**Biotype**	**Biotype**	
O395	C	C	classical	classical	8
C6706	T	T	El Tor	El Tor	8
N16961	T	T	El Tor	El Tor	8
MQ1795	C	C	classical	El Tor	13
BAA-2163	C	C	classical	El Tor	8

Table 1: Biotype Dependent Genetic Distinctions of *Vibrio cholerae* Reference Strains. Shown in this table are the DNA base changes and relative positions in the genes *ctxB* and *tcpA*. WT classical O395 and WT El Tor strains N16961 and C6706 are commonly used biotype reference strains. MQ1795 and BAA-2163 are known El Tor variants.

Assay	Application	Medium Selection	Incubation	Expected Results				
				O395	C6706	N16961	MQ1795	BAA-2163
2.3) Growth Curve[27]	Determines doubling times of various V. cholerae strains	1.2) Liquid LB Broth	up to 30 h (37 °C with aeration)	~2 h	ND*	~1 h	~1 h	ND*
3.1) PCR Based Genetic Screen using ctxB and tcpA[8]	Differentiates between classical and El Tor biotype backgrounds of ctxB	N/A	N/A	classical	El Tor	El Tor	classical	classical
	Differentiates between classical and El Tor biotype backgrounds of tcpA	N/A	N/A	classical	El Tor	El Tor	El Tor	El Tor
3.2) Polymyxin B Resistance[21]	Sensitivity to the antibiotic polymyxin B	1.5) LB agar plates supplemented with polymyxin B	18 h (37 °C)	-	+	+	+	+
3.2) Citrate Metabolism[22]	Ability to metabolize citrate as sole carbon source	1.6) Minimal citrate medium agar plates	24 h (37 °C)	-	+	+	+	+
3.2) Casein Hydrolysis[23]	HapR-regulated protease activity	1.7) Milk agar plates	18 h (37 °C)	-	-	+	+	+
3.2) Hemolysis[23]	Measures hemolytic activity	Blood agar plates	48 h (37 °C)	Gamma	Beta	Beta	Beta	Beta
3.3) Motility[23]	Measures degree of motility	1.8) Motility agar plates	14-24 h (37 °C)	10 mm	15 mm	21 mm	25 mm	29 mm
3.4) Voges-Proskauer[21]	Measures ability to ferment glocose and produce acetoin as a byproduct	1.9) Liquid Voges-Proskauer medium	up to 4 h (room temperature)	-	+	+	-	-
Note: "ND*" denotes not determined; "+" denotes a positive result; "-" denotes a negative result								

Table 2: Summary of Genetic and Phenotypic Assays Used for *Vibrio cholerae* Biotype Distinction. This table summarizes the various genetic and phenotypic assays, applications, and expected results collectively used to differentiate between classical and El Tor biotypes used in this study. Protocol numbers and references to specific protocols are indicated in the Assay column. "ND*" denotes *Not Determined;* "+" denotes a positive result; "-" denotes a negative result.

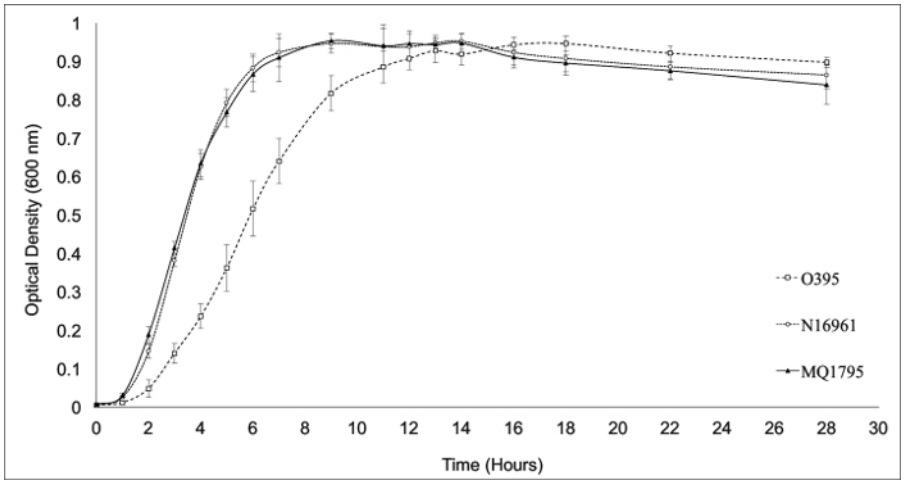

Figure 1: *V. cholerae* Growth Curve of WT Classical O395, WT El Tor N16961, and El Tor Variant MQ1795. Growth rates of biotype reference strains (WT classical O395 and WT El Tor N16961) and representative El Tor Variant MQ1795, grown in LB broth with aeration at 37 °C, were analyzed by measuring the OD_{600} every hour beginning at T_0. Growth curves were performed on 8 independent experimental replicates, with each replicate representing an independent culturing event for each trial. WT El Tor strain N16961 and El Tor variant MQ1795 demonstrated shorter doubling times (~1 h and ~1 h, respectively) relative to WT classical strain O395 (~2 h), as observed by a longer doubling time. Please click here to view a larger version of this figure.

Figure 2: Determining Polymyxin B Resistance Using LB Agar Supplemented with Polymyxin B. Resistance to the peptide antibiotic polymyxin B was determined by the ability to grow on LB agar supplemented with 50 IU/µL polymyxin B. WT classical strain O395 showed no growth on agar supplemented with polymyxin B and was considered sensitive to the antibiotic. While WT El Tor strains (C6706 and N16961) and representative El Tor variants (MQ1795 and BAA-2163) exhibited growth in the presence of the antibiotic and were considered resistant to polymyxin B. Plates were incubated at 37 °C for 18 h. Please click here to view a larger version of this figure.

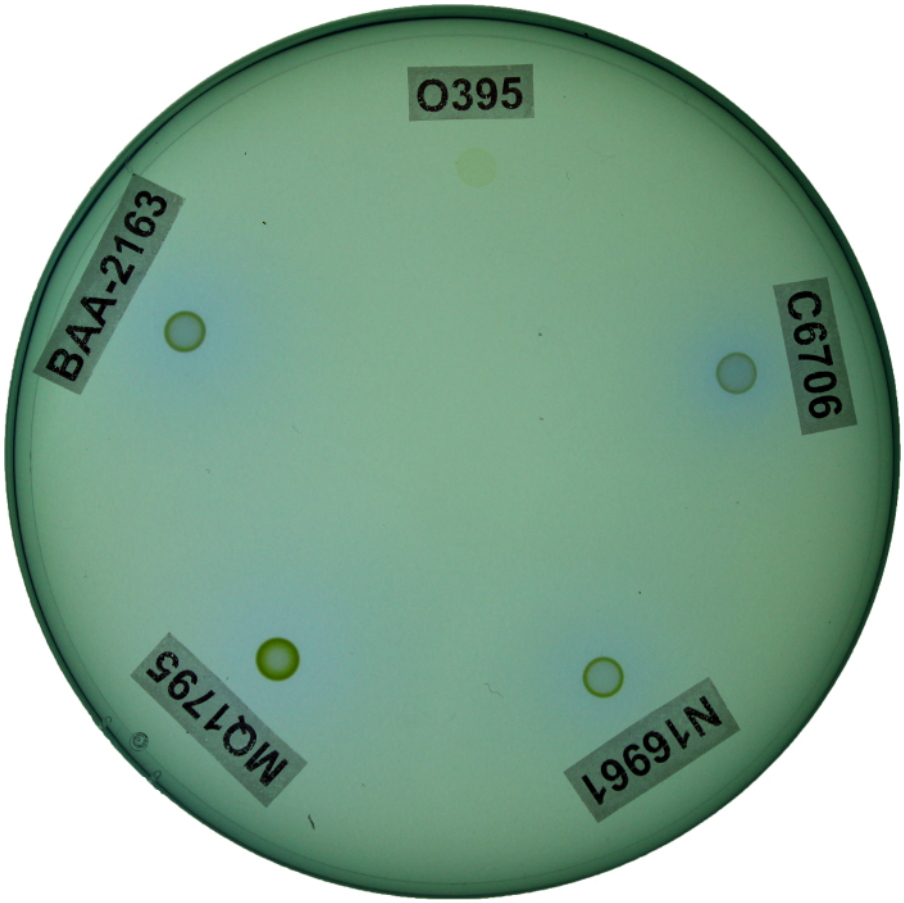

Figure 3: Measuring Citrate Metabolism Using Minimal Citrate Media. The ability to utilize citrate as a sole carbon source was determined by the isolate's ability to grow on minimal citrate media. WT classical strain O395 did not grow on minimal citrate media (negative). Growth was evident by all WT El Tor strains (C6706 and N16961) and representative El Tor variants (MQ1795 and BAA-2163), which demonstrated the ability to utilize citrate as a sole carbon source (positive). Plates were incubated at 37 °C for 18 h. Please click here to view a larger version of this figure.

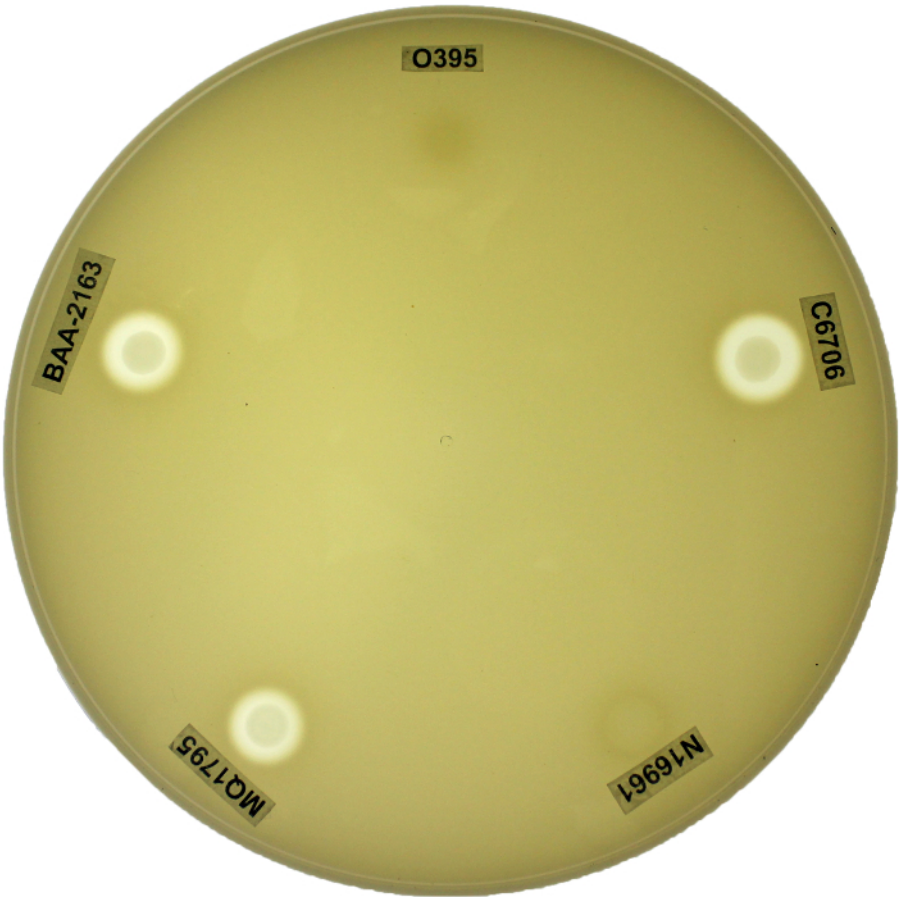

Figure 4: Measuring HapR-regulated Proteolytic Casein Hydrolysis Using Milk Agar Media. Casein hydrolysis through HapR-regulated protease activity was determined by a visual zone of clearance surrounding the point of inoculation on milk agar. Strains containing a non-functional HapR, such as WT classical strain O395 and WT El Tor strain N16961, did not produce a zone of clearance surrounding the point of inoculation (*hapR*-negative). WT El Tor strain C6706 and representative El Tor variants (MQ1795 and BAA-2163) contain a functional HapR, which can be visualized as varying sized zones of clearance (*hapR*-positive). Plates were incubated at 37 °C for 18 h. Please click here to view a larger version of this figure.

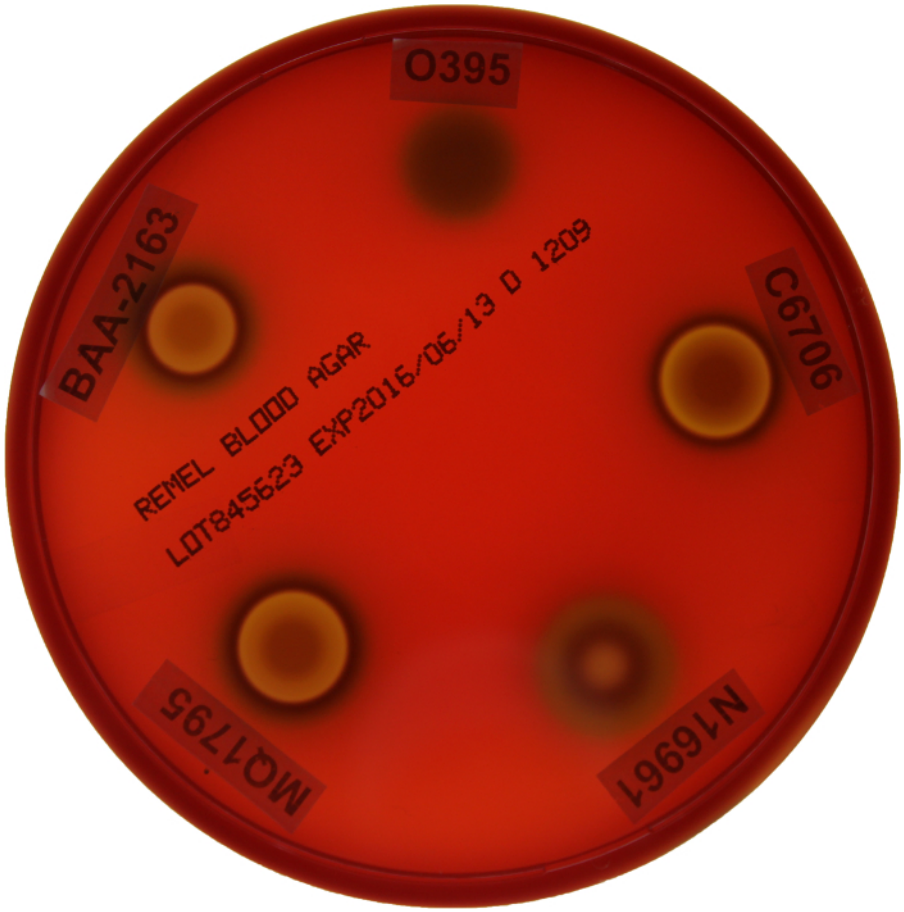

Figure 5: Measuring Hemolytic Activity Using Blood Agar Media. Hemolytic activity was measured using agar plates supplemented with sheep's blood. WT classical strain O395 does not secrete enzymes that lyse red blood cells (γ-hemolytic). WT El Tor strains (N16961 and C6706) and representative El Tor variants (MQ1795 and BAA-2163) secrete hemolytic enzymes, which resulted in a translucent zone of clearance surrounding the point of inoculation (β-hemolytic). Plates were incubated at 37 °C for 48 h. Please click here to view a larger version of this figure.

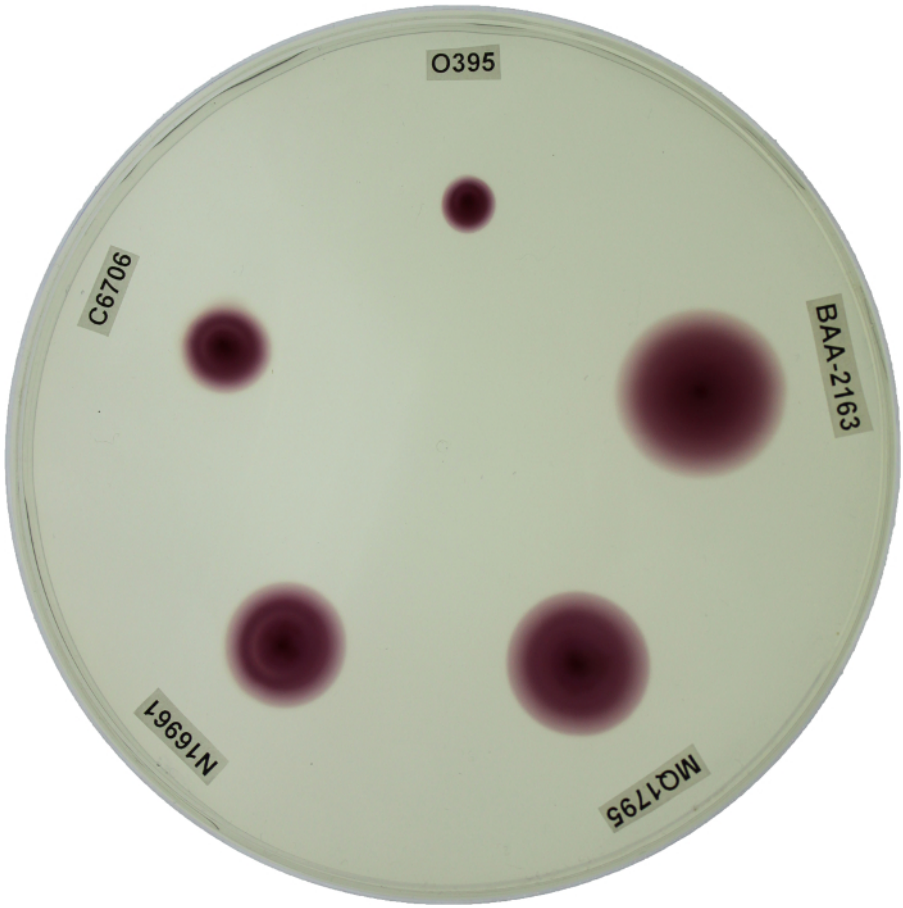

Figure 6: Determining Motility Using Motility Agar Plates. The zone of motility was indicated by a visual color change of the salt TTC which turns from clear to red when metabolized, indicating where the bacteria have moved. WT classical strain O395 (10 mm) and WT El Tor strain C6706 (15 mm) demonstrated minimal motility, while El Tor strain N16961 (21 mm) and representative El Tor variants (MQ1795 (25 mm) and BAA-2163 (29 mm)) demonstrated hyper-motility relative to O395 and C6706. Plates were incubated at 37 °C for 18 h. Please click here to view a larger version of this figure.

Figure 7: Voges-Proskauer Assay. Acetoin production via glucose fermentation was determined using the Voges-Proskauer assay. WT classical strain O395 and representative El Tor variants (MQ1795 and BAA-2163) did not produce acetoin as a result of glucose fermentation (negative). WT El Tor strains (N16961 and C6706) produced the byproduct acetoin and can be visualized by a deep red color change (positive). Tubes were incubated at room temperature for 4 h. Please click here to view a larger version of this figure.

Discussion

Of the over 200 identified *V. cholerae* serogroups, only O1 and O139 have epidemic potential. The O1 serogroup can be divided into two biotypes: classical and El Tor. However, hybrid strains, termed El Tor variants[13,17], have emerged that possess the El Tor biotype background, and harbor classical characteristics[8,9,10,11,12,13,14,15,16,17]. The protocols described in this manuscript are designed to provide investigators, interested in characterizing and/or distinguishing various clinical and non-clinical isolates of *V. cholerae*, with a dependable multi-assay identification system. Use of a dependable multi-assay identification system as an alternative is an improvement over previously established single assay identification systems and labor intensive genetic screens. All genotypic and phenotypic assays should include WT classical strain O395 and WT El Tor strains C6706 and N16961 for comparison (**Table 1**). While the classical and El Tor biotype strains are included for reference, isolates, such as MQ1795 and BAA-2163 included in our studies, can display phenotypic profiles from either biotype (**Figure**

2, **Figure 3**, **Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**; **Table 2**), illustrating the need for analysis of multiple genotypic and phenotypic traits for dependable characterization. All protocols should be carried out aseptically[26] at room temperature unless otherwise specified. *V. cholerae* is a Biosafety Level 2 (BSL-2) pathogen that is the etiological agent of the potentially fatal gastrointestinal disease cholera; proper handling and disposal of all materials and waste products must be enforced per institutional, local, state, and federal regulations.

Preparation of all media and reagents must be carried out using analytical grade reagents and ultrapure water deionized to a minimum sensitivity of 18 MΩ-cm. Prior to processing, media should be prepared and allowed to sufficiently dry (1-2 days); plates are considered sufficiently dry when no residual liquid is present on the surface of the agar. Due to the motile range and zones of clearance surrounding bacterial growth, motility and milk plates should be prepared in large Petri dishes (150 mm x 15 mm) up to 50 mL per plate, and can be stored for up to 1 week wrapped in plastic, lid-side down at 4 ºC. Additionally, the agar concentration of the motility plates can be adjusted to slow down motility; however, it is not recommended to exceed 3 g of agar per 500 mL solution, as this will significantly reduce overall motility such that differences will not be observable. All other plate media should be prepared to approximately 25 mL per plate in standard sized Petri dishes (100 mm x 15 mm) and can be stored for up to six months wrapped in plastic, lid-side down at 4 ºC. For preparation of polymyxin B plates, it is important to allow molten media to cool after autoclaving prior to adding polymyxin B, as excessive heat can degrade antibiotics. Polymyxin B plates can be stored for up to 3 months wrapped in plastic, lid-side down at 4 ºC. Assays discussed in this manuscript require a day for single colony growth (12-16 h), an additional day for growth of overnight cultures (12-16 h), and a third day for considerations of genotypic (~4 h for chromosomal DNA isolation and ~3 h for PCR) or phenotypic assays (18-48 h; **Table 2**). Prior to the biochemical analysis of phenotypic assays described in this manuscript, cultures should be washed in 1x PBS to prevent residual culture media carryover from skewing results. Additionally, polymyxin B, citrate, protease activity, hemolysis, and motility assays may be inoculated simultaneously using a single washed overnight culture. When spotting plated media, splatter of cultures may occur and can result in cross contamination between strains. To prevent splatter, avoid completely ejecting culture from the pipette, instead stop ejecting the culture at the first stop of the pipette. Additionally, allow spots to fully absorb into the agar surface prior to incubation, and take care not to puncture the agar, which can affect the results. Phenotypic assays resulting in zones of clearance (**Figure 4**; **Figure 5**; **Table 2**) and/or motility (**Figure 6**; **Table 2**) surrounding the point of inoculation, should be maximally spaced to prevent merging of areas of clearance or growth, respectively, upon which the respective diameters can be measured for comparative analysis. After indicated incubation times, isolates can be imaged and analyzed relative to the biotype reference strains (WT classical O395, WT El Tor C6706, and WT El Tor N16961).

The PCR-based genetic screens through sequencing outlined in this manuscript can be used to identify the isolate's biotype background with respect to *ctxB* and *tcpA*, following initial PCR amplification. Standard sequencing guidelines require a specific amount of PCR product depending on the size of the amplified region (580 bp for *ctxB* and 1420 bp for *tcpA*), and for setting up the reactions, established protocols can be followed. Briefly, individual forward and reverse sequencing reactions should be prepared with 3-5 pmol of primer/reaction, and the volume should be adjusted to 20 µl with sterile ultrapure water in a 1.5 mL microcentrifuge tube. For aid in primer design for both PCR amplification and sequencing, the NCBI primer design tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/ can be utilized. Successful amplification and sequencing of *ctxB* and *tcpA* has been accomplished using the following primers (5'→3'): *ctxB*-forward GGGAATGCTCCAAGATCATCGATGAGTAATAC, *ctxB*-reverse CATCATCGAACCACAAAAAAGCTTACTGAGG, *tcpA*-forward CCGCACCAGATCCACGTAGGTGGG, *tcpA*-reverse GTCGGTACATCACCTGCTGTGGGGGCAG. It should be noted that the entire coding region of *ctxB* is conserved across both biotypes except for base positions 115 and 203 (both cytosine in classical and thymine in El Tor). Additionally, in *tcpA*, multiple base changes are conserved among the biotypes that differ across the two biotypes (**Table 1**), which can be used to help distinguish between biotypes.

In many laboratory studies involving the model organism *V. cholerae*, proper maintenance and culturing techniques are critical[27]. The growth rates of commonly used *V. cholerae* biotype reference strains, such as WT classical strain O395 and WT El Tor strain N16961, can provide useful insight into the characterization of El Tor variant isolates (**Figure 1**; **Table 2**). Because of the varying growth rates across *V. cholerae* isolates, it is important to take spectrophotometer absorbance readings every hour until the cultures reach maximum turbidity during the late stationary phase. Mid-to-late death phase may not be visualized due to a plateau in turbidity resulting from excess cellular debris. A 1:4 dilution of culture to sterile LB broth should be performed to obtain a complete growth curve and maintain precision of the instrument, as absorbance readings peak at an $OD_{600} \approx 1.0$. When performing growth curves on multiple strains, absorbance readings should be timed approximately 5 minutes apart for each strain to ensure consistency between readings. Understanding *V. cholerae* biotype growth rates are crucial for many investigations including virulence gene expression studies, analysis of metabolic activities, and proper culturing and storage conditions. For subsequent analysis, overnight cultures should be processed in the late log to early stationary phase of growth (12-16 h) to maximize cell growth yet maintain cellular integrity.

Culturing conditions for classical and El Tor biotype strains are similar, however, analysis of virulence gene expression in *V. cholerae* requires biotype-specific virulence inducing conditions[19]. The two main virulence factors CT and TCP, are controlled by the master regulator ToxT, and are optimally expressed under specific growth conditions; for example, under El Tor virulence inducing conditions ToxT can be analyzed by processing whole cell extract (WCE), or the cell pellet, at 3.5 h, while CT and TCP expression can be analyzed by processing the cell-free supernatant and WCE at 7.5 h, respectively[8,20]. The varying genotypic and phenotypic traits demonstrated throughout this manuscript indicate how diverse *V. cholerae* biotypes can be, and illustrate the need for an alternative to previously used single-assay biotype characterization.

Disclosures

The authors have nothing to disclose.

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