Identification Guide

Isolation and Identification of *Vibrio* Species from Culture-independent Diagnostic Test Positive Specimens

APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

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Contents

Introduction

Purpose

A recommended workflow for the culture and isolation of *Vibrio* species from human fecal specimens identified as positive for *Vibrio* species or *Vibrio cholerae* by a culture-independent diagnostic test (CIDT).

Background

The following is a procedure for the culture and isolation of *Vibrio* species from human feces that have tested positive for *Vibrio* by a CIDT. *Vibrio* is a common etiologic agent of bacterial diarrhea and can cause severe or fatal infections. The genus *Vibrio* contains over 100 species with 12 species that can cause clinical diseases. *V. cholerae* has historically caused large outbreaks with severe disease and still causes outbreaks in countries with limited public health infrastructure. *Vibrio* is generally located in aquatic environments and major risk factors for *Vibrio* infections include consumption of raw seafood (especially oysters) and exposure to coastal waters.

V. cholerae is divided into more than 200 serogroups but only O1 and O139 cause large epidemics and pandemic cholera.[1] Serogrouping to determine O1 and O139 is useful for further differentiating *V. cholerae* to determine whether an isolate belongs to a potential epidemic lineage. O1 can be further divided into three serotypes: Inaba, Ogawa, and the rarely seen Hikojima serotype.[1] *Cholerae* toxin (*ctxAB*) is a major virulence factor and impacts the severity of disease.

Sensitive and specific laboratory methods for the isolation and identification of *Vibrio* are key to monitoring and control efforts. Advances in clinical diagnostic testing have led to the widespread use of CIDTs in clinical laboratories, which are used to provide rapid and sensitive testing for enteric pathogens. One challenge with current CIDT methods that include *Vibrio* species as a target is the high amount of apparent false positives seen for *Vibrio*.[2,3] Specimens that test positive for *Vibrio* by a CIDT but are unable to be cultured may represent false positives, especially if the patient lacks traditional *Vibrio* exposures such as consumption of raw or under-cooked seafood, foreign travel, or exposure to coastal waters. Positive *Vibrio* CIDT results that do not yield *Vibrio* after reflex culture when the case does not have traditional *Vibrio* risk factors should be viewed with suspicion. Current CIDT assays target *Vibrio* species while a minority include a target specific for *V. cholerae*.

While CIDTs can be a benefit to patient care by providing faster results, they do not yield isolates which are necessary for further characterization by public health laboratories. Isolates are critical for subtyping, monitoring disease trends, and identifying antibiotic resistance in laboratories. In recent years, public health laboratories have received a drastic increase in the volume of primary specimens that tested positive for a reportable enteric pathogen by a CIDT platform, including specimens submitted as *Vibrio* positive. The goal of this document is to provide public health laboratories with methods to isolate and identify *Vibrio* spp. from stool specimens that have tested positive for *Vibrio* species by a CIDT as efficiently and cost effectively as possible.

Fecal specimens are the preferred source for diagnosis of infectious diarrhea. However, the recovery of enteric pathogens from feces is often complicated by multiple factors including prior antibiotic treatment, transport stress, intermittent shedding of pathogens in the feces, and a low inoculum of *Vibrio* bacteria in relation to other enteric flora. These factors necessitate the use of culture algorithms that employ selective enrichment and the use of selective and differential media. An additional challenge with culturing *Vibrio* is that under periods of stress they can enter a dormant phase called viable but non-culturable (VBNC).[4] In this scenario it can be useful to use enrichment broth to promote *Vibrio* growth.

Selective enrichment suppresses fecal flora while allowing the target pathogen to grow. Selective media can also utilize phenotypic characteristics to preliminarily differentiate potential pathogens from fecal flora.

The number of media and reagents that can be used to identify *Vibrio* continues to expand. It is important to routinely review scientific literature and manufacturer product announcements to identify products that could improve *Vibrio* recovery.

Specimen Collection and Transport

The preferred specimen for *Vibrio* culture is fresh stool collected in transport media such as Cary-Blair (CB).[5] Unpreserved stool and fecal or rectal swabs submitted without transport media are not preferred but may also be acceptable; however, these specimens should be considered for rejection if transit time exceeds limits stated by the receiving laboratory (often two hours after specimen collection). If the unpreserved sample cannot reach the laboratory within the specified time, it is recommended that the raw stool cultures be transferred into a non-nutritive buffered transport medium (such as CB) and stored at 4°C to preserve pathogen viability. Glycerol can be toxic to *Vibrio* making buffered glycerol saline (BGS) unsuitable as a transport media for *Vibrio*.[6] Suspect *Vibrio* specimens collected in CB or other non-inhibitory transport media should be shipped at 2–8°C, though shipping at ambient temperature is acceptable, especially for species such as *V. vulnificus* which can enter a viable but non-culturable state when storage conditions are too cold.[6] Ideally, specimens should be received as quickly as possible, not exceeding four days since collection as isolate recovery may decline. At the public health laboratory, testing for *Vibrio* should begin as soon as reasonably possible. Specimens should be held at 2–8°C until culture is completed.

Materials and Supplies

Media

- **Preferred Selective/Differential Media**:
  Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS)
- **Less-Selective Media** (use at least one):
  Blood Agar (BAP), MacConkey Agar (MAC)
- **Enrichment Broth** (optional):
  Alkaline Peptone Water (APW)
- **Biochemicals for screening:**
  - Oxidase.
  - Other potential screening options include: salt tolerance broths, Triple Sugar Iron (TSI), Motility Indole Ornithine (MIO), spot indole and Lysine Iron Agar (LIA)
- **Biochemicals for identification:**
  See Appendix E (page 12) for a full list of biochemicals.

Other Supplies

- Inoculating loops
- Applicator swabs
- Transfer pipettes (for inoculation of APW)
- Supplies for MALDI-TOF (if performed)
- Serotyping regents (if serotyping is performed)
- PCR reagents (if PCR is performed)
- WGS reagents (if WGS is performed)

All testing performed and media used for identification and isolation of *Vibrio* should follow the manufacturer's instructions.

Culture and Identification Workflows

The following describes specific workflows that could be utilized at public health laboratories. The Standard Workflow (Appendix A, page 9) is the most cost-effective but has reduced sensitivity for the detection of *Vibrio*, as it does not utilize an enrichment broth. The Optional Enrichment Broth workflow (Appendix B, page 10) uses more reagents and takes longer but will increase the chances to recover *Vibrio* spp.,[7] especially when there are few organisms present in the stool or if the stool has not been maintained and transported at the optimal conditions. Since *Vibrio* is relatively rare, public health laboratories may consider devoting more resources to isolating the pathogen and should choose the workflow(s) that best fits their surveillance needs and resources. In general, performing MALDI-TOF for species identification will be the most cost-effective identification method, however whole genome sequencing (WGS) can provide important information in addition to species identification. The use of biochemicals for species identification is the least efficient and public health laboratories should consider other methods, depending on the needs for their jurisdiction.

Standard Workflow

See "Appendix A: Standard Workflow Diagram for Isolation and Identification of Vibrio*" (page 9).*

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

1. Inoculate one selective media (TCBS) and at least one less selective media (must include BAP, could also include MAC). *See Additional Comments (page 7) for more information on media considerations.*

Day 1

1. Pick suspicious colonies from TCBS to BAP and incubate overnight. Check primary TCBS for suspicious colonies at both 24 and 48 hours.
2. If there are no suspicious colonies, consider:
   a. Oxidase testing on a sweep from the BAP. If the sweep is oxidase positive, perform additional isolation to attempt to identify oxidase positive colonies.
   b. Adding the specimen to an enrichment broth.
   See Optional Enrichment Broth Workflow (page 6) or Appendix B (page 10).

Day 2

1. From the BAP, perform a limited number of screening assays before moving to identification methods.
   - At a minimum: perform oxidase testing.
   - Other screening tests to consider are: salt tolerance broths, TSI, API20E, MIO, spot indole and LIA. See *Appendix E (page 12)* for *Vibrio* biochemical results.
2. If initial screening results are consistent with *Vibrio* spp., perform methods to identify the species of *Vibrio*.
   Note: The most common methods used to identify Vibrio at public health laboratories are MALDI-TOF, WGS, biochemicals, and genus and species-specific PCR. See Appendix F (page 13) for more information about Vibrio identification methods.

Optional Enrichment Broth Workflow

*See "**Appendix B: Optional Enrichment Broth Workflow Diagram for Isolation and Identification of* Vibrio*" (page 10)*.

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

1. Inoculate APW with stool (not to exceed more that 10% of the volume of the broth) and allow it to grow for 6-8 hours at 35-37 °C.[8]
2. If APW cannot be streaked after 6-8 hours of incubation:
   a. Subculture at 18 hours to a fresh tube of APW
   b. Incubate 6-8 hours, then streak onto TCBS.[3]

   Note: Vibrio tend to grow at greatest density at the top of liquid media, so when streaking plates from APW it is important to take the inoculum from the top 1 cm of the APW.[9]

Day 1

1. Inoculate one selective media (TCBS) and at least one less selective media (must include BAP, could also include MAC).

Day 2

1. Pick suspicious colonies to BAP and incubate overnight. TCBS should be checked for suspicious colonies at both 24 and 48 hours.
2. If there are no suspicious colonies, consider performing an oxidase sweep from the BAP.

Day 3

1. From the BAP, perform a limited number of screening assays before moving to identification methods.
   - At a minimum: perform oxidase.
   - Other screening tests to be considered are growth on: salt tolerance broths, TSI, API20E, MIO, spot indole and LIA.
2. If initial screening results are consistent with *Vibrio* spp., perform methods to identify the species of *Vibrio*.

   *Note: The most common methods used to identify Vibrio to the species level at public health laboratories are MALDI-TOF, WGS, biochemicals, and genus and species-specific PCR. See **Appendix F (page 13)** for more information about Vibrio identification methods.*

Serogrouping

Serogrouping of *V. cholerae* provides information that can be useful for investigations and epidemiological purposes. It is recommended that public health laboratories perform serogrouping for O1 and O139 and test for the *ctxAB* toxin, resources permitting. The serogroup can also be determined by WGS. Serogrouping antisera is available from commercial suppliers. For additional inquiries involving antisera, contact CDC's Enteric Reference Laboratory at entericreferencelab@cdc.gov.

Additional Comments

- Some strains of *Vibrio* are inhibited on MAC.[2]
- TCBS is formulated for the growth of *Vibrio*. *Grimontia* species and some strains of *Vibrio* do not grow well on TCBS so it is important to also use a non-selective media such as MAC or BAP.[1]
- Oxidase should not be performed on colonies derived from TCBS and MAC as it can yield incorrect results.[1] Instead, oxidase testing can be performed on colonies from non-selective media such as BAP.
- It is important to note colony morphology and color as color indicates sucrose fermenting properties on TCBS which are important for identifying genus and species.
- Some strains of *Vibrio* produce multiple colony morphologies, particularly on TCBS.[1]
- The ability to differentiate some species can be problematic due to similar phenotypic characteristics. There are also other stool pathogens (*Aeromonas* and *Grimontia*) that have similar transmission, clinical significance and colony morphology as *Vibrio* species.
- Some strains of *V. mimicus* carry the *ctxA* gene.[1]
- Some public health laboratories perform PCR for certain *Vibrio* species directly from the stool. If the PCR is negative the specimen is reported as a negative. If the specimen is positive, the specimen is inoculated on a TCBS in order to identify *Vibrio* colonies.
- Some species such as *V. harveyi, V. metschnikovii, Grimontia* spp., and *Photobacterium* spp. preferentially grow at 30 °C.
- The recommendations made here are based on the best information and data available for isolation and identification of *Vibrio* in public health laboratories. Please contact entericreferencelab@cdc.gov or foodsafety@aphl.org with any comments or questions.

References

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2 Decuir, M., et al., Evidence of False Positivity for Vibrio Species Tested by Gastrointestinal Multiplex PCR Panels, Minnesota, 2016-2018. Open Forum Infect Dis, 2021. 8(6): p. ofab247.

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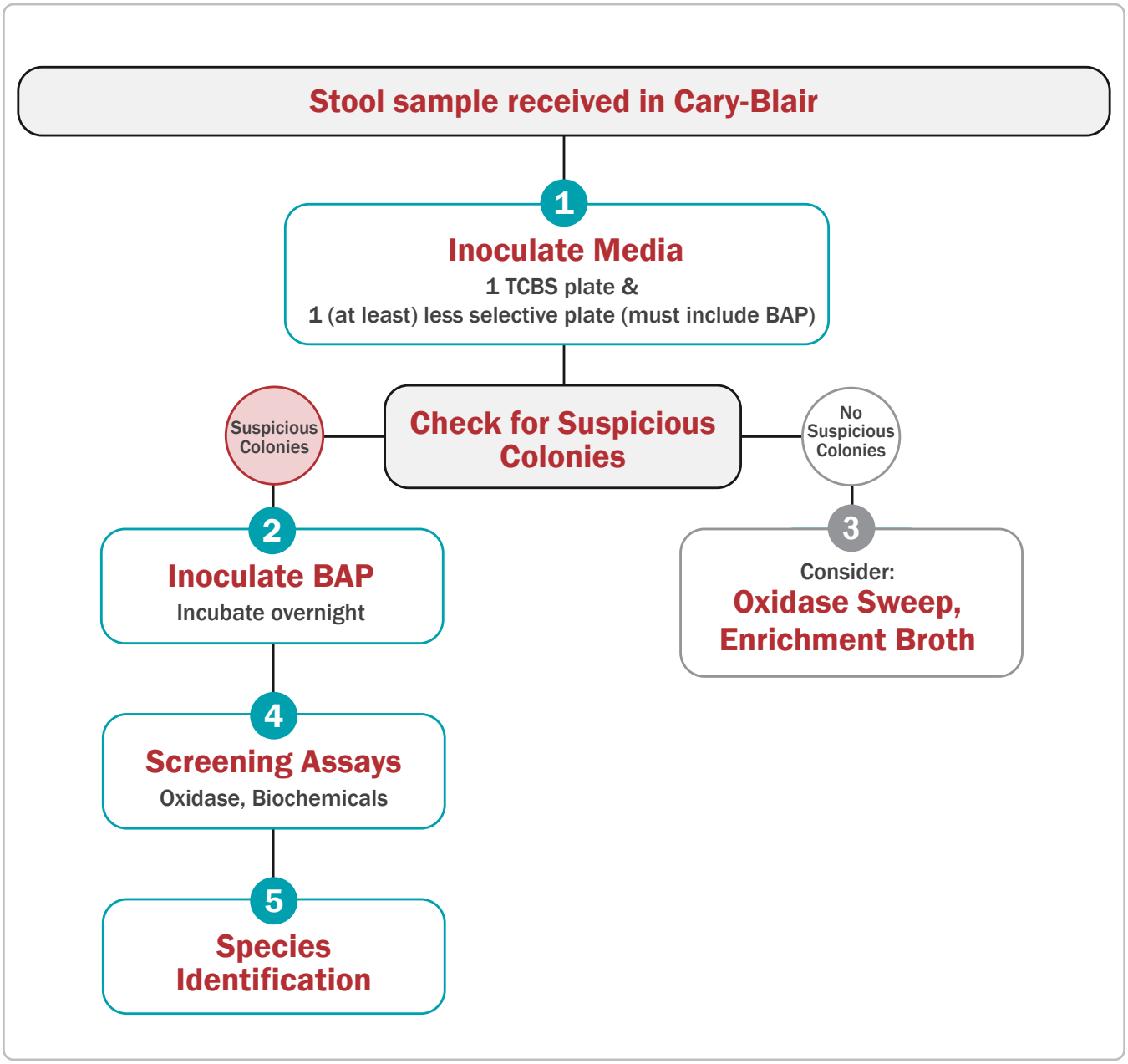
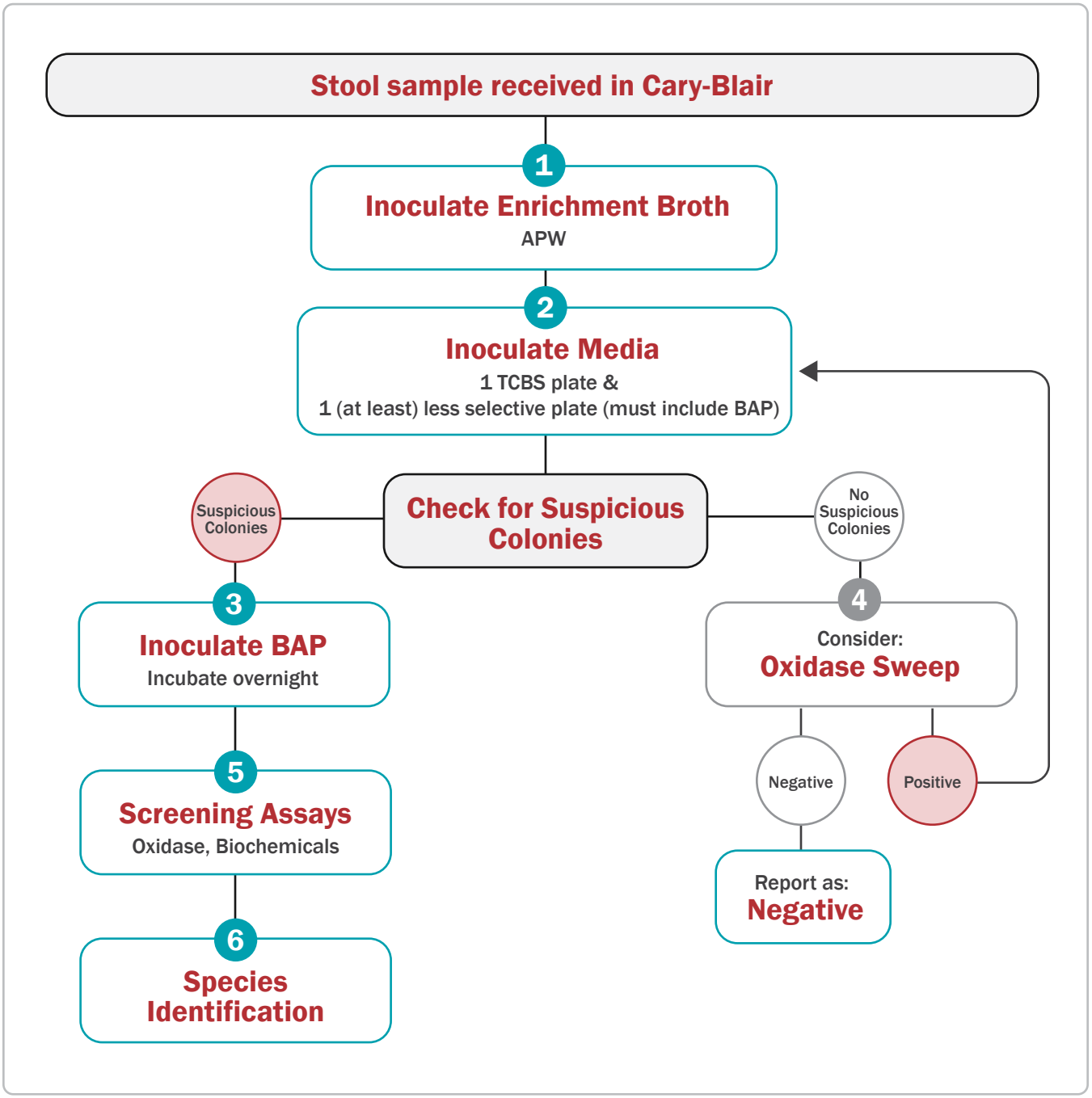
Appendix

Appendix A: Standard Workflow Diagram for Isolation and Identification of *Vibrio*



Appendix B: Optional Enrichment Broth Workflow Diagram for Isolation and Identification of *Vibrio*



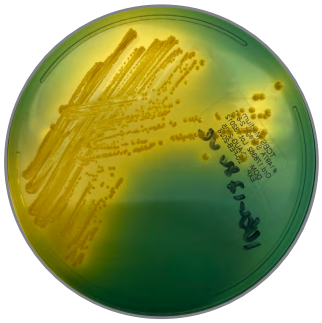
Appendix C: Important Morphologic Characteristics of *Vibrio* spp. on Recommended Media

Media	*V. cholerae*	*V. parahaemolyticus*
TCBS*	Yellow, shiny colonies; 2-3 mm in diameter	Green colonies
BAP	Beta-hemolytic colonies	Alpha- or beta-hemolytic colonies
MAC	Non-fermenting, colorless to light pink colonies; 1-3 mm in diameter	Small, non-fermenting, smooth colonies

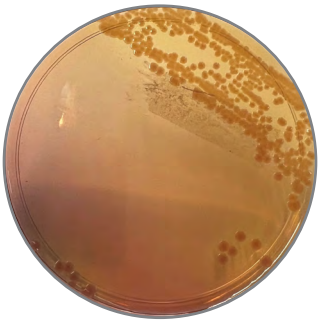
* *V. cholerae, V. metoecus, V. paracholerae, V. arriae, V. fluvialis*, and *V. alginolyticus* all produce yellow colonies on TCBS, while *V. parahaemolyticus, V. mimicus*, and most strains of *V. vulnificus* produce green colonies.

Appendix D: Morphology of *Vibrio* spp. on Non-selective and Differential Media

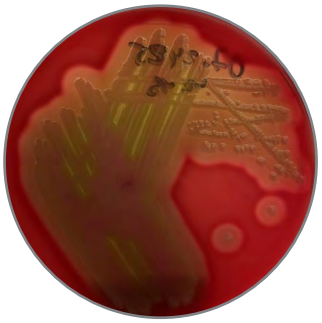
1.
***V. cholerae* on TCBS agar**



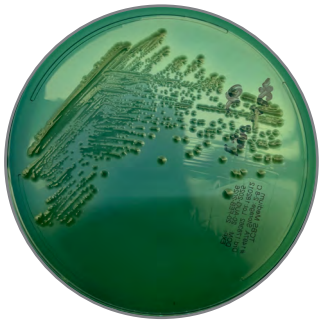
2.
***V. cholerae* on MAC agar**



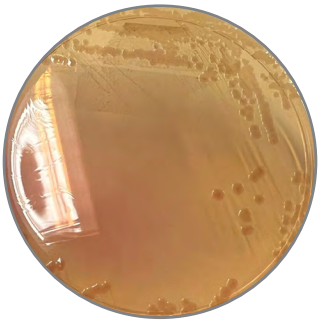
3.
***V. Cholerae* on BAP**



4.
***V. parahaemolyticus* on TCBS agar**



5.
***V. parahaemolyticus* on MAC agar**



6.
***V. parahaemolyticus* on BAP**

Appendix E: Typical Biochemical Reactions for *Vibrio* spp. and Other Similar Species[1,10]

The numbers in the table below are the percentage of strains that are positive within24 hours. "+" means that most strains are positive, while "-" means most strains are negative. Percentages are historical and have not been updated to account for new species that may exhibit similar biochemical profiles.

Biochemicals	*V. cholerae*	*V. parahaemolyticus*	*Aeromonas* spp.	*Grimontia* spp.
Nutrient broth with 0% NaCl	100	0	100	0
Nutrient broth with 6% NaCl	50	99	-	83
Oxidase	+	+	+	+
Indole	+	+	96	-
Motility	99	99	100	0
22°C Gelatin Hydrolysis	90	95	+	0
Arabinose	0	80	84	97
Salicin	1	1	76	0
Glucose (gas)	0	0	92	0
Lactose	7	1	16	0
Maltose	+	+	100	+
Mannitol	+	+	96	-
Sucrose	100	1	100	0
Arginine Dihydrolysis	0	0	+	0
Lysine Decarboxylase	99	100	+	0
Ornithine Decarboxylase	99	95	0	0
Voges-Proskauer	75	0	88	0
O/129 susceptibility	99	20	0	40

Appendix F: Methods for *Vibrio* Identification in Public Health Laboratories

Techniques	Pros	Cons
Biochemicals	Commonly used to ID pathogens.	Time consuming, costly, includes many biochemicals.
MALDI-TOF	Quick, cost effective and already performed in many laboratories.	Current IVD libraries contain limited *Vibrio* species. Likely would need to create an expanded database.
PCR	Quick, cost effective and already routinely performed in public health laboratories.	No current FDA-approved assays. Detects limited specific species so assays may not detect some species.
WGS	Routinely performed in public health laboratories. Can provide an identification of *Vibrio* to the genus and species. Detects the serogroup, serotype, toxins and will yield a subtype. WGS is the established subtyping method for PulseNet.	Takes several days which can delay reporting results. Requires in-house validation to report results.

Biochemicals

The traditional method for identifying *Vibrio* species is through biochemicals. Identification using only biochemicals can take a long time and be costly, as many biochemicals may be needed to identify a species. In addition, some biochemicals are not commonly used in some public health laboratories.

MALDI-TOF

Current FDA-approved MALDI-TOF instruments include limited *Vibrio* species. The Bruker IVD library includes *V. parahaemolyticus* and *V. vulnificus* while the Vitek MS IVD library includes *V. alginolyticus, V. cholerae, V. fluvialis, V. metschnikovii, V. mimicus, V. parahaemolyticus* and *V. vulnificus*. *V. cholerae* is included in the Bruker SR database. It may be desirable for public health laboratories to create their own expanded *Vibrio* database; CDC's Enteric Disease Laboratory Branch (EDLB) Reference Laboratory can provide panels of additional *Vibrio* species for database expansion.

PCR

There is not an FDA-approved PCR specific to *Vibrio*. Some public health laboratories have created and validated laboratory-developed tests (LDT) for *Vibrio* and select *Vibrio* species. Public health laboratories have the equipment and experience necessary to perform PCR. Public health laboratories that have created LDTs for *Vibrio* PCR are only able to identify specific species. Some *Vibrio* species can exchange genes with other species making PCR challenging.

WGS

PulseNet 2.0 can identify 15 *Vibrio* species using WGS data, including *V. alginolyticus, V. cholerae, V. cidicii, V. cincinnatiensis, V. fluvialis, V. furnissii, V. harveyi, V. metoecus, V. metschnikovii, V. mimicus, V. navarrensis, V. paracholerae, V. parahaemolyticus, V. tarriae* and *V. vulnificus*. *Grimontia hollisae* is a former *Vibrio* spp. (*V. hollisae*) also identifiable in PulseNet 2.0. In addition, WGS data can be used to determine the serogroup, toxin genes, and provide subtype data, specifically for PulseNet surveillance species *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. Public health laboratories would need to perform necessary validations according to their laboratory protocols to report results to the submitter. While public health laboratories routinely perform WGS, it is a costly process.

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