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Sea Grant Maryland

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Methods for the Detection of *Vibrio* Species

Laboratory Protocol for the Enumeration of Total and Pathogenic *Vibrio* Species in Oyster Meat

Vibrio bacteria naturally live in certain coastal waters and are frequently isolated from a variety of raw seafood, particularly oysters. About a dozen *Vibrio* species can cause human illness, known as vibriosis. The most common species causing human illness in the United States (US) are *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* (https://www.cdc.gov/vibrio/).

Vibrio species are common causes of foodborne illnesses in many Asian countries, including China and Japan, and are recognized as the leading cause of human bacterial gastroenteritis associated with seafood consumption in the United States (Su and Liu*, 2007). The Centers for Disease Control and Prevention (CDC) estimates that vibriosis causes 80,000 illnesses each year in the US. About 65% (52,000 out of 80,000) of these illnesses are reported to be the result of eating contaminated food (https://www.cdc.gov/vibrio/).

Most people become infected by eating raw or undercooked shellfish, particularly oysters. Symptoms include acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, and abdominal cramps. Certain *Vibrio* species can also cause a skin infection when an open wound is exposed to brackish or salt water.

*: Su, Y-C and Liu, C (2007). *Vibrio parahaemolyticus*: a concern of seafood safety-A Review, Food Microbiology 24 (2007): 549-558.

The methods described in this report are designed to enumerate *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* in oysters.

The analytical procedures are based on a combination of procedures from the following sources:

Cook, D.W, A. DePaola, and S.A. McCarthy, 2000. Direct plating procedure for the enumeration of total and pathogenic *Vibrio parahaemolyticus* in oyster meats. FDA/Gulf Coast Seafood Laboratory.

DePaola, A., G.M Capers, and D. Alexander, 2004. Chapter 9 *Vibrio*. In: FDA Bacteriological Analytical Manual (BAM). Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm (Accessed on November 20, 2017).

Jones, J.L., Y. Hara-Kudo, J. A. Krantz, R.A. Benner, A.B. Simth, T.R., Dambaugh, J.C. Bowers and A. DePaola, 2012. Comparison of molecular detection methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Food Microbiology*. 30:105-111.

BAX® System Real-Time PCR Assay for *Vibrio*. Available at: https://www.hygiena.com/bax-vibrio-rt.html (Accessed on November 20, 2017)

1. Equipment and Materials

1. Stainless steel mesh hand gloves
2. Temperature probe
3. Autoclave for sterilizing media
4. Incubator 35ºC ±2ºC
5. Refrigerator at 2-8º C
6. Balance, capacity≥ 2 kg, 0.1 g sensitivity
7. Balance, capacity≥ 100 g, 0.01 g sensitivity
8. Water bath, or shaking environmental chamber, capable of maintaining 42ºC, 54ºC, and 55ºC ±0.1ºC (for DNA probe)
9. Orbital shaker, small for use at room temperature
10. Microwave oven, 1000 watts
11. Transfer loops (1mm to 10mm)
12. Pipetter, single channel, 2-20 µl
13. Pipetter, single channel, 20-200 µl
14. Pipetter, single channel, 100 -1000 µl
15. Pipet Tips
16. Sterile 100x15 mm petri dishes, glass or plastic
17. Fine-tip waterproof marker
18. Blender and sterile blender jars
19. Brushes for scrubbing oysters
20. Shucking knives
21. Sterile cell spreader
22. Sterile cluster tubes
23. Serological pipettes (5, 10 & 25 ml)
24. Pipet-aid
25. Culture dish, glass, 100 x 20 mm
26. Tweezers for filter handling
27. Whatman #541 filters, 85 mm
28. Washing container, wide mouth jar, plastic with screw cap, 500 ml
29. Plastic Whirl-Pak® bags, (4.5" x 9") (NASCO B00736WA)
30. Disposable 15 ml tubes (optional)
31. 100 ml bottles (optional)
32. Heating blocks (37 and 95 °C)
33. Cooling blocks (2-8°C)
34. DuPont BAX® Q7 System
35. DuPont BAX® Decapping Tool

2. Media and Reagents

1. Tryptic Soy Agar with 2% NaCl (TSA-2%)
2. Alkaline peptone water (APW)
3. Phosphate buffer saline (PBS)
4. Thiosulfate citrate bile salts sucrose (TCBS) agar
5. Modified cellobiose polymyxin colistin (mCPC) agar
6. Cellobiose colistin (CC) agar
7. T1N3 (for Vp probe)
8. VVA (for Vv probe)
9. Lysis solution (0.5M NaOH 1.5M NaCl)
10. Ammonium acetate buffer (2M)
11. Standard Saline Citrate (SSC) Solution (20x)
12. 1xSSC
13. 3xSSC
14. 5xSSC
15. Stock Proteinase K (ProK) Solution
16. Hybridization buffer
17. 1xSSC/SDS solution
18. NBT/BCIP solution
19. BAX system *Vibrio* real-time PCR Assay kit containing protease, lysis buffer and PCR tablets
20. 2% Sodium chloride (NaCl)
21. api®20E

3. Control Cultures

The Food and Drug Administration's Bacteriological Analytical Manual (BAM) recommends using positive and negative controls for all phenotypic and genotypic assays to ensure appropriate interpretation of the reactions.

4. Experimental Protocol

4.1. Collection of Oyster Samples

1. Plan collection of oyster samples so that analysis will be initiated the same day as harvest. If this cannot be done, oysters must be held at a temperature of ≤10°C, but not frozen, and analysis initiated within 36 hours (h) of harvest.
2. Oysters may be harvested by dredging or tonging. Immediately after harvest, oysters should be culled and rinsed to remove excess mud. For each sample, place 13 to 15 oysters in a plastic bag, label bag, and place oysters

in an insulated chest, using bubble wrap or other inter-packing material to avoid direct contact of oysters with wet ice or blue ice. Keep the oysters in the chest until delivered to the analytical laboratory.

4.2. Sample Processing and Serial Dilutions

1. Upon receipt in the laboratory, verify that the internal temperature of at least one representative oyster is ≤10°C.
2. To check the temperature, wear stainless steel mesh hand gloves (optional) and using a knife, pop the hinge of the animal and insert a temperature probe into the meat. If the temperature is >10°C, do not initiate analysis of the sample. Investigate the reason for high temperature and take corrective action. [**Note**: If oysters were harvested within 3 h of examination, sufficient cooling time may not have elapsed and oyster may be >10°C. Consider such samples as acceptable.]
3. Wash oysters with a stiff brush under cold running tap water to remove mud and debris. Place oyster on absorbent paper to drain. [**Note**: The remaining steps in this section must be carried out without delay.]
4. Shuck 10 to 12 oysters (include meat and shell liquor) into a sterile tared blender jar. If needed to facilitate homogenizing, add an equal weight of sterile phosphate buffered saline (PBS). The total volume in the blender jar should completely cover the blades.
5. Blend for 60-120 sec.
6. Prepare a 1:10 dilution volumetrically in PBS (e.g., 1 g into 9 ml if undiluted, 2 g into 8ml if diluted 1:2 in blender).
7. Additional 10-fold dilutions can be prepared volumetrically (i.e. 1 ml of 1:10 dilution of oyster meat to 9.0 ml of PBS for a 1:100 dilution). The number of dilutions to be prepared depends on anticipated *Vibrio* density. [**Note**: BAM recommends PBS, rather than alkaline peptone water (APW) for blending and subsequent dilutions to prevent growth during the processing. The use of APW is acceptable so long as this entire process is completed in <15 min.]

4.3. Serial Dilutions for Most Probable Number (MPN)

Use APW in 10 ml volumes. For 3-tube MPN analysis, inoculate 3 tubes at each dilution as follows:

1. Weigh 1 g of the homogenate (2 g if diluted 1:2 for blending) into each of tubes containing 10 mL of APW. This represents 1 g of oyster meat.
2. Inoculate 1 ml portions of the 1:10 dilution into each of tubes containing 10 ml of APW (equivalent to 0.1 g of oyster meat).
3. Inoculate 1 ml portions of the 1:100 dilution into each of 3 tubes containing 10 ml of APW (equivalent to 0.01 g of oyster meat).
4. Inoculate 1 ml portions of the 1:1,000 dilution into each of 3 tubes containing 10 mL of APW (equivalent to 0.001 g of oyster meat). [**Note:** Use at least 3 consecutive dilutions, inoculate 1 ml aliquots from each dilution into 3 APW tubes. If high numbers of *V. parahaemolyticus* are expected, the examination may start at the 1:10 dilution of oyster meat homogenate and dilutions of up to 1:1,000,000 may be needed.]
5. Incubate MPN tubes at 35±2°C for 18-24 h.

4.4. Preparation of Direct Plates for Colony Hybridization

1. Using a balance having a sensitivity of 0.01 g, weigh 0.1±0.01 g of homogenate (0.2±0.01 of 1:2 homogenate, if diluent used for blending) onto the surface of each of three dried T1N3 plates for total and pathogenic Vp and onto one VVA plate for Vv. Immediately spread the sample over the surface of the plate with a sterile spreading rod and continue spreading each plate until all liquid is absorbed.

2. Place 100 µl of the 1:10 dilution onto the surface of dried T1N3 and/or VVA plates, for 0.01g samples. Immediately spread the sample over the surface of each plate with a sterile spreading rod. Plate additional dilutions as needed, based on the expected vibrio levels.
3. Incubate all plates at 35°C for 18-24 h.

4.5. Examination of MPN Tubes and Plating

1. Examine the MPN tubes and record the number of turbid tubes (those showing growth). Table 1 provides an example of a record sheet.
2. Streak a loopful of all turbid (positive) tubes (between 1 mm and 10 mm) onto TCBS agar for the isolation of *V. parahaemolyticus* and *V. cholerae* and mCPC or CC agar for the isolation of *V. vulnificus*.
   [**Note**: According to the BAM, a 3-mm loopful should be streaked from the top 1 cm of APW tubes containing the three highest dilutions of sample showing growth onto TCBS agar. However, streaking from all positive tubes is recommended. In addition, streaking between 1 mm and 10 mm of the APW tubes, with any volume loop is acceptable as long as isolation can be achieved.]
3. Incubate TCBS plates at 35±2°C for 18-24h. Incubate mCPC or CC plates at 35-40°C for 18-24h.

4.6. Examination of Spread Plates and Colony Hybridization

1. Label a Whatman #541 filter for each sample plate to be hybridized with a fine point permanent marker. Place the filter, labeled side down, on the surface of the plate with colony growth. Use a spreading rod to press the filter directly against the agar surface to insure colonies are transferred. Filters may be lifted as soon as filter is wet or may remain on the plate for up to 30 min.
2. Place 1 ml of lysis solution in the center of an inverted 100x15 mm petri dish lid (one for each filter to be lysed). Remove the #541 filter with colonies from the T1N3 plate and place colony side up over the lysis solution. Position filter to exclude all air bubbles between the filter and the dish. The process is intended to wet the entire filter with the lysis solution. Do not let the lysis solution run over the surface of the filter.
3. Place petri dishes (maximum of six) with filters in microwave and heat on full power (1000 watts or less) for 30 sec/filter. Filters should be completely dry, but not brown. If needed, heating time may be extended.
4. Place 4 ml of ammonium acetate buffer for each filter to be neutralized in a clean plastic "washing" container. Add the filters one at a time, insuring that each filter is saturated before adding the next. Let filters remain in buffer for 5 min. at room temperature with swirling on the orbital shaker.
5. Decant the ammonium acetate buffer from the "washing" container. Add 10 ml of lx SSC solution per filter. Swirl container for 1 to 2 min. Decant the liquid and rinse a second time by adding 10 ml of 1x SSC per filter to the container and swirling for 1 to 2 min. Decant solution.
6. Continue with probing of filters or place filters, colony side up, onto absorbent paper and allow to air dry at room temperature. Once dry, filters can be stored indefinitely in plastic bags until ready to probe.
7. Add 10 ml of lxSSC and 20 µl stock ProK into the "washing" container for each filter to be treated. Warm to 42°C and add filters one at a time to ensure that each is saturated with the solution before the next is added. Incubate with shaking (~50 spm) in an environmental chamber or in a water bath, static or shaking, at 42°C for 30 min.
8. Rinse filters three times in lxSSC (l0 ml/filter) for 10 min. at room temperature in the "washing" container with shaking on the orbital shaker at ~125 rpm.

Filters can be dried, as above at this point and stored indefinitely or you can proceed with hybridization.

9. Place one to five appropriately marked filters in a plastic (Whirl Pak® 4.5"x9") bag marked to receive the appropriate gene probe. Add a control strip to each bag (see section 4.7.). Add 10 ml of hybridizing buffer to each bag. Weigh corner of bags so that they remain completely submerged, but are free to move with shaking. Incubate with shaking (~50 spm) in an environmental chamber or in a water bath, static or shaking, at 42°C for 30 min. Alternatively, you can use a 'washing' container to probe up to 20 filters at a time with hybridizing buffer at the ratio of 10 ml per five filters.
10. Pour buffer from the bag or container and add 10 ml of fresh pre-warmed hybridizing buffer. Add 5 picamoles of probe per 10 ml of fresh hybridizing buffer and mix gently. If bags are used, ensure air bubbles are excluded during closure. Incubate 1 h at 54°C for tlh, tdh, and trh in bags separated by probe type; incubate 1 h at 55°C for vvh.
11. If bags are use, remove filters and place those probed with tdh, into separate "washing" containers from the other probes. If containers are used, decant hybridization solution.
    a. Rinse tlh and trh filters two times with 1xSSC/SDS (l0 ml filter) for 10 min. in bath at 54°C with shaking. Rinse tdh filters two times with 3xSSC/SDS (l0 ml filter) for 10 min. in bath at 54°C with shaking.
    b. Rinse vvh filters two times with 1xSSC/SDS (l0 ml filter) for 10 min. in bath at 55°C with shaking.
12. Filters can now be combined into one "washing" container. Rinse five times for 5 min. in 1x SSC (l0ml/filter) at room temperature with shaking on the orbital shaker.
13. Into a petri dish (100x20 mm), add 20 ml of NBT/BCIP solution and add up to five filters. Incubate with shaking at room temperature or at 35°C for faster results. Cover to omit light during incubation. Check development of positive control on the control strip every hour. The NBT/BCIP solution is considered hazardous, so ensure proper handling and disposal.
14. Stop the development reaction when control colonies are developed by placing filters into a "washing" container with distilled or deionized water (10 ml/filter). Rinse three times for 10 min. each time.
15. Place filters on absorbent paper in the dark to dry. Count and record the number of colony blots that develop a bluish-gray or dark purple color. Colony blots that are colorless, yellow, gray or light brown are negative. Filters should be stored in the dark to prevent color change. Filters may be photocopied or scanned into a computer to produce a permanent record.
16. Express results in colony-forming units (CFU) per gram of oysters in sample. Each colony on a 0.1 g filter represents 10 CFU/g and each colony on a 0.01 g filters represents 100 CFU/g.

4.7. Control Strips

Control strips are prepared using *V. vulnificus*, a *tdh*- strain of *V. parahaemolyticus*, and a *tdh*+ strain of *V. parahaemolyticus*. These strains are spot inoculated in multiple lines on a T1N3 plate and incubated overnight. Colony lifts are made from the plate and the filters are lysed. The filters are cut into strips so that each strip contains all of the three controls. Control strips can be mass produced, dried and stored for later use. A control strip should be used in each bag of filters being probed. The expected reactions from the controls are as follows.

Culture	*tlh* Probe	*tdh* Probe
V. vulnificus	−	−
tdh⁻ *V. parahaemolyticus*	+	−
tdh+ *V. parahaemolyticus*	+	+

4.8. BAX® System Lysis Sample Preparation and Real-Time PCR Assay

1. Turn on the BAX® heating blocks. Create a rack file in the BAX® system software.
2. Prepare lysis reagent by adding 150 µl of protease to one 12 ml bottle of lysis buffer.
3. Transfer 200 µl of lysis reagent to each cluster tube.
4. Add a 5 µl aliquot from each turbid (positive) MPN tube to the corresponding cluster tube.
5. Place the rack of cluster tubes on a pre-warmed heating block at 37°C for 20 min.
6. Transfer rack to a pre-warmed heating block at 95°C for 10 min.
7. Transfer the cluster tubes to a cooling block and allow to cool for 5 min.
8. Initialize the BAX® System Instrument.
9. Place *Vibrio* Real-Time PCR Assay tubes in a chilled PCR cooling block.
10. Remove the caps from the strip of tubes with the decapping tool and transfer 30 µl of lysate from the cluster tubes to corresponding PCR tubes.
11. Place new optical caps on the strip of tubes and secure tightly. Repeat for all samples.
12. Load tubes into the BAX® System Q7 instrument and run the program.
13. After approximately 1 h, read the results and record all positives for each species *V. cholerae, V. parahaemolyticus* and *V. vulnificus*.
14. Each MPN tube which produces a BAX® positive result for a particular organism is considered positive for that organism.
15. Determine the MPN/g for each organism independently using the standard 3-tube MPN table in the FDA-BAM (Available: https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm

4.9. Plate Examination, Isolate Purification and Biochemical Identification

1. Examine the TCBS plates after incubation at 35 ±2°C overnight.
   Typical ***V. parahaemolyticus*** colonies will appear as follows:
   - TCBS agar- round, opaque, green or bluish colonies, 2 to 3 mm in diameter.
     Note: Interfering competitive *V. alginolyticus* colonies are, large, opaque, and yellow.

   Typical ***V. cholerae*** colonies will appear as follows:
   - TCBS-large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries.

   Typical ***V. vulnificus*** colonies will appear as round, flat, opaque, yellow, 1 to 2 mm in diameter on mCPC or CC agars.
2. Streak typical colonies from TCBS and mCPC or CC agar plates to TSA- 2% agar (or other non-selective agar) to purify isolates. **Note**: For biochemical identification, colonies from selective agar must be streaked to a non-selective agar for purity.
3. Incubate overnight at 35±2°C and proceed with identification using a single isolated colony.
4. A microtiter plate can be inoculated with isolates from selective media for biochemical identification, confirmation by DNA probe, and/or freezer storage. For more information about biochemical identification of *V. parahaemolyticus* and confirmation by DNA probe, please refer to the FDA BAM.

4.10. Biochemical Identification using api®20E

1. From the overnight cultures streaked on TSA-2%, prepare a cell suspension of an isolated colony using 2% NaCl.
2. Prepare the api®20E tray by distributing approximately 5 ml of distilled water evenly among the honeycombed wells.
3. Remove the api®20E test strip and place in the tray for inoculation.
4. Follow the manufacturer's instructions for inoculation and analysis.
5. Repeat for each TSA plate, recording the sample information on the elongated flap.
6. Incubate at 35±2°C for 18-24 h. Record the numerical profile for each sample and use to identify the isolate using the online database apiweb.
7. Each MPN tube from which a confirmed *Vibrio* spp. is isolated is considered positive for that organism.
8. Determine the MPN/g for each organism independently using the standard 3-tube MPN table in the FDA-BAM (Available: https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm)

Media & Reagents

Note: Distilled or deionized water (dH_2O) may be used for all media and reagents.

*Denotes media that can be found in the FDA BAM Media Index https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm055778.htm

Trypticase Soy Agar with 2 % NaCl (TSA-2%)

Trypticase peptone	15 g
Phytone peptone	5 g
NaCl	5 g
Agar	15 g
dH_2O	1000 ml

The first four ingredients represent the contents of commercial TSA. Prior to heating, an additional NaCl (20 g per liter) is required to achieve a final concentration of 2% NaCl. Heat with gentle agitation to dissolve agar. Autoclave for 15 min at 121°C. Pour into sterile petri dishes. Store up to 6 months.

Alkaline Peptone Water (APW)*

Peptone	10 g
NaCl	10 g
dH_2O	1000 ml

Dissolve ingredients. Adjust pH with NaOK so that value after sterilization is 8.5±0.2. Dispense 100 ml amounts in media bottles and autoclave for 15 min at 121°C. Store under refrigeration. Discard after 90 days.

Phosphate Buffer Saline (PBS)*

NaCl	7.65 g
Na_2HPO_4, anhydrous	0.724 g
KH_2PO_4	
dH_2O	1000 ml

Dissolve ingredients in dH_2O. Adjust pH to 7.4 (with 1 N NaOH). Autoclave for 15 min at 121°C. Store at room temperature. Discard after 90 days.

Thiosulfate citrate bile salts sucrose (TCBS) agar*

Yeast extract	5 g
Peptone	10 g
Sucrose	20 g
Sodium thiosulfate $5H_2O$	10 g
Sodium cholate	3 g
Oxgall	5 g
NaCl	10 g
Ferric citrate	1 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	15 g
dH_2O	1000 ml

Prepare in flask at least 3 times larger than required volume of medium. Add ingredients to warm dH_2O and heat to dissolve. Bring to boil and immediately remove from heat. **DO NOT AUTOCLAVE!** Cool to 50°C and pour into petri dishes. Dry the plates overnight or at 37-45°C before use.

Modified Cellobiose polymyxin colistin (mCPC) Agar*

Solution 1

Peptone	10 g
Beef extract	5 g
NaCl	20 g
1000x Dye stock solution	1 ml
dH_2O	900 ml

Add ingredients, Adjust to pH 7.6. Cool to 48-55°C.

1000x Dye stock solution

Bromothymol blue	4 g
Cresol red	4 g
Ethanol, 95%	100 ml

For consistent medium color, use dye solution rather than repeatedly weighing out dry dyes. Dissolve dyes in ethanol for 4% (w/v) solution. Using 1 ml of this solution per liter of mCPC agar gives 40 mg bromothymol blue and 40 mg cresol red per liter.

Solution 2

Cellobiose	10 g
Colistin	400,000 units
Polymyxin B	100,000 units
dH_2O	100 ml

Dissolve cellobiose in dH_2O by heating gently. Cool. Add antibiotics. Add Solution 2 to cooled solution 1, mix, and dispense into petri dishes. Final color, is dark green to green-brown.

Note: This medium is very inhibitory and does not require autoclaving. Medium may be stored two weeks at refrigeration temperatures.

Cellobiose colistin (CC) agar*

Solution 1

Peptone	10 g
Beef extract	5 g
NaCl	20 g
1000x Dye stock solution	1 ml
dH_2O	900 ml

Add ingredients. Adjust to pH 7.6. Cool to 48-55°C.

1000x Dye stock solution	
Bromothymol blue	4 g
Cresol red	4 g
Ethanol, 95%	100 ml

For consistent medium color, use dye solution rather than repeatedly weighing out dry dyes. Dissolve dyes in ethanol for 4% (w/v) solution. Using 1 ml of this solution per liter of mCPC agar gives 40 mg bromothymol blue and 40 mg cresol red per liter.

Solution 2

Cellobiose	10 g
Colistin	400,000 units
dH_2O	100 ml

Dissolve cellobiose in dH_2O by heating gently. Cool. Add antibiotics and filter sterilize. Add solution 2 to cooled solution 1, mix, and dispense into petri dishes. Final color is dark green to green-brown.

Note: This medium is very inhibitory and does not require autoclaving. Medium may be stored two weeks at refrigeration temperatures.

T1N3 Agar

Tryptone	10 g
NaCl	30 g
Agar	20 g
dH_2O	1000 ml

Adjust pH to 7.2 before heating. Autoclave, cool and pour into plates. Immediately after plates solidify, package in plastic bags and store under refrigeration. Discard after 90 days.

Vibrio vulnificus* Agar (VVA)

Solution 1

Peptone	10 g
NaCl	30 g
100x Dye stock solution**	10 ml
Agar	25 g
dH_2O	900 ml

Add ingredients, Adjust to pH 8.2. Boil to dissolve agar. Autoclave for 15 min at 121°C. Temper to 55°C.

100x Dye stock solution	
Bromothymol blue	0.6 g
Ethanol, 70%	100 ml

For consistent medium color, use dye solution rather than repeatedly weighing out dry dyes.

Solution 2

Celliobiose	10 g
dH_2O	100 ml

Dissolve cellobiose in dH_2O by heating gently. Cool and filter sterilize. Add solution 2 to cooled solution 1, mix, and dispense into petri dishes. Final color is light blue. Medium may be stored under refrigeration temperatures for two weeks.

Lysis solution
(0.5M NaOH 1.5M NaCl)

NaOH	20 g
NaCl	87 g
dH_2O	1000 ml

Store at room temperature. Discard after 90 days.

Ammonium acetate buffer

Ammonium acetate	154 g
dH_2O	1000 ml

Store at room temperature. Discard after 90 days.

Standard Saline Citrate (SSC) Solution

20x SSC

NaCl	175.4 g
Sodium Citrate $2H_2O$	88.2 g
dH_2O	1000 ml

Dissolve in 800 ml dH_2O and adjust to pH 7 with 10 N NaOH. Bring to volume of 1000 ml. Store at room temperature. Discard after 90 days.

5x SSC

20x SSC	25 ml
dH_2O	75 ml

3x SSC

20x SSC	150 ml
dH_2O	850 ml

1x SSC

20x SSC	50 ml
dH_2O	950 ml

Dilute prior to use. Discard 1xSSC after 1 day.

Stock Proteinase K (ProK) solution

Add 5 ml dH_2O to 100 mg bottle of proteinase K. The stock ProK solution will contain 20 mg/ml. Divide into 200 µl aliquots and store frozen at -20°C.

Hybridization buffer

Bovine Serum Albumin (BSA) (Fraction V Powder)	0.5 g
Sodium Dodecyl Sulfate (SDS) (Sodium Lauryl Sulfate)	1.0 g
Polyvinylpyrrolidone (PVP-360)	0.5 g 5x
SSC	100 ml

Store at 4°C for up to one week. Warm to 54°C before use.

1xSSC/SDS solution

Sodium dodecyl sulfate (SDS)	10.0 g
1x SSC	1.0 L

Store at room temperature. Discard after 90 days.

NBT/BCIP solution
Just before use, dissolve 2 NBT/BCIP ready-to- use tablets (Boehringer Mannheim, Cat. No.1697471) in 20 ml of dH_2O in a glass petri dish. Discard after one use.

THL-L AP probe
Probe sequence is 5'XAAAGCGGATTATGC AGAAGCACTG 3' where X=alkaline phosphatase conjugated 5' Amine-C6. Probe available from LGC Biosearch Technologies, Voldbjergvej 16, DK-8240 Risskov, Denmark; Phone: +45 8732 3000; Email: eu@biosearchtech.com; https://eu.biosearchtech.com/.
Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. **DO NOT FREEZE**. Shelf life is unknown, but exceeds one year.

TDH-M2 AP-probe
Probe sequence is 5'XGGTTCTATTCCAAG TAAAATGTATTTG 3' where X=alkaline phosphatase conjugated 5' Amine-C6. Probe available from LGC Biosearch Technologies, Voldbjergvej 16, DK-8240 Risskov, Denmark; Phone: +45 8732 3000; Email: eu@biosearchtech.com; https://eu.biosearchtech.com/.
Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. **DO NOT FREEZE**. Shelf life is unknown, but exceeds one year.

TRH AP-probe
Probe sequence is 5' XACTTTGCTTTCAGT TTGCTATTGGCT 3' where X=alkaline phosphatase conjugated 5' Amine-C6. Probe available from LGC Biosearch Technologies, Voldbjergvej 16, DK-8240 Risskov, Denmark; Phone: +45 8732 3000; Email: eu@biosearchtech.com; https://eu.biosearchtech.com/.
Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. **DO NOT FREEZE**. Shelf life is unknown, but exceeds one year.

VVHA AP-probe
Probe sequence is 5' XGAGCTGTCACGG CAGTTGGAACCA 3' where X=alkaline phosphatase conjugated 5' Amine-C6. Probe available from LGC Biosearch Technologies, Voldbjergvej 16, DK-8240 Risskov, Denmark; Phone: +45 8732 3000; Email: eu@biosearchtech.com; https://eu.biosearchtech.com/.
Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. **DO NOT FREEZE**. Shelf life is unknown, but exceeds one year.

Calculation of probe amount
Data sheets provided with each batch of probe will show the concentration of the probe in nanomoles (nmol) and the total volume of probe shipped. (µl probe equal to 5 picomoles)= (volume in µl x 5) ÷ (concentration in nmol x 1000).

Table 1. Shellfish *Vibrio parahaemolyticus* (Vp) MPN results					
Shellfish	**(APW) (+/-)**	**BAX Real Time-PCR**		**Total Vp Culture**	
		Vp (+/-)	**IAC (+/-)**	**TCBS (T/NT*)**	**Probe (+/-)**
1g A					
1g B					
1g C					
-1 A					
-1 B					
-1 C					
-2 A					
-2 B					
-2 C					
-3 A					
-3 B					
-3 C					
-4 A					
-4 B					
-4 C					
-5 A					
-5 B					
-5 C					
-6 A					
-6 B					
-6 C					
PC					
NTC					
MPN/g					
Date					
Analyst					
Notes:					

*T=typical colony growth; NT=non-typical colony growth

Figure 1. Flowchart of hands-on training workshop for enumeration of total pathogenic *Vibrio* species in oyster meat

See the pages 14-17.

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Figure 1. Flowchart of hands-on training workshop for enumeration of total pathogenic *Vibrio* species in Oyster Meats

Sample Processing and Serial Dilutions

(Steps in this section must be carried out without delay)

1. Shuck 10 to 12 oysters (include meat and shell liquor) into a sterile tared blender jar.

   Note: If needed to facilitate homogenizing, add an equal weight of sterile PBS[1]. The total volume in the blender jar should completely cover the blades (This will be a 1:2 dilution.).
2. Blend for 60 -120 sec.
3. Prepare a 1:10 dilution volumetrically in PBS[1] (e.g., 1 g into 9 ml if undiluted, 2 g into 8 ml if diluted 1:2 in blender).
4. Additional 10-fold dilutions can be prepared volumetrically (i.e. 1 ml of 1:10 dilution of oyster meat to 9 ml PBS[1] for a 1:100 dilution). The number of dilutions to be prepared depends on anticipated *Vibrio* density.

   Note: FDA Bacteriological Analytical Manual (BAM) recommends PBS[1] rather than APW[2] for blending and dilutions to prevent growth during processing. APW[2] can be used if the entire process is completed in ≤15 min.

Serial Dilutions for Most Probable Number (MPN)

Use APW[2] in 10 ml volumes. For 3-tube MPN analysis, inoculate 3 tubes at each dilution as follows:

- Weigh 1 g of the homogenate (2 g if diluted 1:2 for blending) into each of 3 tubes containing 10 ml of APW[2]. This represents 1 g of oyster meat.
- Inoculate 1 ml portions of the 1:10 dilution into each of 3 tubes containing 10 ml of APW[2] (equivalent to 0.1 g of oyster meat).
- Inoculate 1 ml portions of the 1:100 dilution into each of 3 tubes containing 10 ml of APW[2] (equivalent to 0.01 g of oyster meat).
- Repeat volumetric transfers for subsequent dilutions.

Note: Use at least 3 consecutive dilutions; inoculate 1 ml aliquots from each dilution into 3 APW[2] tubes. If high numbers of *V. parahaemolyticus* (Vp) are expected, the examination may start at 1:10 dilution of oyster meat homogenate and dilutions up to 1:1,000,000 may be needed.

Incubate MPN tubes at 35±2°C for 18-24 h.

Preparation of Direct Plates for Colony Hybridization

1. Weigh 0.1±0.01 g of homogenate (0.2 ±0.01 g of 1.2 homogenate, if diluent used for blending) onto the surface of each of three dried T1N3[3] plates for total and pathogenic Vp and onto one (1) VVA[4] plate for *V. vulnificus* (Vv).
2. Immediately spread the sample over the surface of the plate with a sterile spreading rod and continue spreading each plate until all liquid is absorbed.
3. Place 100 µl of the 1:10 diltion onto the surface of a dried T1N3[3] and/or VVA[4] plates, for 0.01 g samples. Repeat step 2 above. Plate additional dilutions as needed, based on the expected *Vibrio* levels.
4. Incubate all plates at 35±2°C for 18-24 h.

Examination of MPN Tubes, Plating and BAX System Real-Time PCR Assay

Examination of Spread Plates and Colony Hybridization

Examination of MPN Tubes, Plating and BAX System Real-Time PCR Assay

Examination of MPN Tubes and Plating

1. Examine MPN tubes and record the number of turbid tubes (those showing growth) on the record sheet.
2. Streak a loopful of all turbid (positive) tubes onto TCBS[5] agar for the isolation of Vp and *V. cholerae* (Vc) and mCPC[6] or CC[7] agar for the isolation of Vv.

   Note: According to the BAM, a 3-mm loopful should be streaked from the top 1 cm of APW[2] tubes containing the three highest dilutions of sample showing growth onto TCBS[5] agar. However, streaking from all positive tubes is recommended. In addition, streaking between 1-mm and 10-mm of the APW[2] tubes, with any volume loop is acceptable as long as isolation can be achieved.

3. Incubate TCBS[5] plates at 35 ±2°C for 18-24 h. Incubate mCPC[6] or CC[7] plates at 35-40°C for 18-24 h.

Plate Examination and Isolate Purification

Examine the TCBS[5], mCPC[6], or CC[7] agar plates for typical growth.

- Typical Vp colonies will appear as round, opaque, green or bluish, 2 to 3 mm in diameter on TCBS[5].

  Note: Interfering competitive *V. alginolyticus* colonies are large, opaque and yellow.

- Typical Vc colonies will appear as large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries on TCBS[5].
- Typical Vv colonies will appear as round, flat, opaque, yellow, 1 to 2 mm in diameter on mCPC[6], or CC[7] agars.

Streak typical colonies fromTCBS[5], mCPC[6], or CC[7] agar plates to TSA-2%[8] agar or other non-selective agar) to purify isolates.

Note: For biochemical identification, colonies from selective agar must be streaked to a non-selective agar for purity.

Incubate overnight at 35 ±2°C.

Biochemical Identification

Refer to the FDA BAM for more information on biochemical identification of Vp and confirmation by DNA probe.

BAX System Lysis and Sample Preparation for Real-Time PCR Assay

Screen all turbid (positive) MPN tubes using BAX® *Vibrio* Real-time PCR kit following the manufacturers guidelines.

Record all positives for each species (Vc, Vp and Vv).

Note: Each MPN tube producing a BAX positive result for a particular organism is considered positive for that organism.

Determine the MPN/g for each organism independently using the standard 3-tube MPN table (refer to the FDA BAM)

Examination of Spread Plates and Colony Hybridization

Preparation of Colony Lifts

1. Label a Whatman #541 filter with a fine point permanent marker for each sample plate to be hybridized. Place the filter label side down on the surface of the plate with colony growth. Use a spreading rod to press the filter directly against the agar surface to insure colonies are transferred. Filters may be lifted as soon as filter is wet or may remain on the plate for up to 30 min.
2. Place 1 ml of lysis solution in the center of an inverted 100x15 mm petri dish lid (one per filter to be lysed). Remove the filter with colonies from the T1N3[3] plate and place colony side up over the lysis solution. Position filter to exclude all air bubbles between the filter and the dish. The process is intended to wet the entire filter with the lysis solution). Do not let the lysis solution run over the surface of the filter.
3. Place petri dishes with filters (maximum of six) in the microwave and heat on full power (1000 watts of less) for 30 sec/filter. Filters should be completely dry, but not brown. If needed, heating time may be extended.
4. Into a clean plastic "washing" container, place 4 ml of ammonium acetate buffer for each filter to be neutralized. Add the filters one at a time insuring that each filter is saturated before adding the next. Let filters sit for 5 min at room temperature with swirling on the orbital shaker.
5. Decant the ammonium acetate buffer from the "washing" container. Add 10 ml of 1xSSC[9] solution per filter. Swirl container for 1 to 2 min.
6. Repeat step 5 and decant the solution.
7. Continue with probing of filters or place filters, colony side up, onto absorbent paper and allow to air dry at room temperature. Once dry, filters can be stored indefinitely in plastic bags until ready to probe.

Probing the Colony Lifts

1. Into the "washing" container, add 10 ml of 1xSSC and 20 µl stock ProK for each filter to be treated. Warm to 42°C and add filters one at a time to ensure that each is saturated with the solution before the next is added. Incubate with shaking (~50 spm) in an environmental chamber or in a water bath, static or shaking, at 42°C for 30 min.
2. Rinse filters three times in 1xSSC[9] (10 ml/filter) for 10 min at room temperature in the washing container with shaking on the orbital shaker at ~125 rpm. Filters can be dried, as above, at this point and stored indefinitely or you can proceed to hybridization.
3. Place one to five appropriately marked filters in a plastic (Whirl Pack 4.5" x 9") bag marked to receive the appropriate gene probe. Add a control strip to each bag. Add 10 ml of hybridizing buffer to each bag. Weigh corner of bags so that they remain completely submerged, but are free to move with shaking. Incubate with shaking (~50 spm) in an environmental chamber or in a water bath, static or shaking, at 42°C for 30 min. Alternatively, a "washing container" can be used to probe up to 20 filters at a time with hybridizing buffer at the ratio of 10 ml per five filters.

Probing the Colony Lifts (cont.) and Control Strips

Probing the Colony Lifts (cont.)

4. Pour buffer from the bag or container and add 10 ml of fresh pre-warmed hybridizing buffer. Add 5 picamoles of probe per 10 ml of fresh hybridizing buffer and mix gently. If bags are used, ensure air bubbles are excluded during closure. Incubate 1 h at 54°C for *tlh*, *tdh*, and *trh* in bags separated by probe type; incubate 1 h at 55°C for vvh.

5. If bags are used, remove filters and place those probes with *tdh* into separate "washing" containers from the other probes. If containers are used, decant hybridization solution.

   a. Rinse *tlh* and *trh* filters twice with 1xSSC/SDS[10] (10 ml/filter) for 10 min in a bath at 54°C with shaking.

   b. Rinse *tdh* filters twice with 3xSSC/SDS[11] (10 ml/filter) for 10 min in a bath at 54°C with shaking.

6. Filters can now be combined into one "washing" container. Rinse five times for 5 min in 1xSSC[9] (10 ml/filter) at room temperature with shaking on the orbital shaker.

7. Into a petri dish (100 x 20 mm), add 20 ml of NBT/BCIP solution[12] and add up to five filters. Incubate with shaking at room temperature or at 35°C for faster results. Cover to omit light during incubation. Check development of positive control on the control strip every hour.

   Note: The NBT/BCIP solution[12] is considered hazardous, so ensure proper handling and disposal.

8. Stop the development reaction when control colonies are developed by placing filters into a "washing" container with distilled or deionized water (10 ml/filter). Rinse three times for 10 min each.

9. Place filters on absorbent paper in the dark to dry. Count and record the number of colony blots that develop a bluish-gray or dark purple color. Colony blots are colorless, yellow, gray or light brown are negative. Filters should be stored in the dark to prevent color change. Filters may be photocopied or scanned into a computer to produce a permanent record.

10. Express results in colony forming units (CFU) per gram of oysters in a sample. Each colony on a 0.1 g filter represents 10 CFU/g and each colony on a 0.01 g filter represents 100 CFU/g.

Control Strips

Prepare control strips using *V. vulnificus*, a *tdh*- strain of *V. parahaemolyticus*, and a *tdh*+ strain of *V. parahaemolyticus*. These strains are spot inoculated in multiple lines on a T1N3[3] plate and incubated overnight. Colony lifts are made from the plate and the filters are lysed. The filters are cut into strips so that each strip contains all of the three controls. Control strips can be mass-produced, dried and stored for later use. A control strip should be used in each bag of filters being probed. The expected reactions from the controls are as follows.

Culture	*tlh* Probe	*tdh* Probe
V. vulnificus	−	−
tdh⁻ *V. parahaemolyticus*	+	−
tdh⁺ *V. parahaemolyticus*	+	+

[1] Phosphate buffer saline;
[2] Alkaline phosphate water;
[3] Tryptone salt agar;
[4] *Vibrio vulnificus* agar;
[5] Thiosulfate citrate bile salts sucrose;
[6] Modified cellobiose polymyxin colistin;
[7] Cellobiose colistin;
[8] Tryptic soy agar + 2% NaCl;
[9] 1x Standard Saline Citrate;
[10] 1x Standard Saline Citrate + Sodium Dodecyl Sulfate (SDS);
[11] 3x Standard Saline Citrate + SDS;
[12] NBT/BCIP tablets + distilled water.