National Antimicrobial Resistance Monitoring System
Seafood Pilot Study Laboratory Protocol

Sample collection:

- Collect seafood from the stores assigned that month for retail meat collection. 2 shrimp, 2 salmon and 2 tilapia.

- Look for raw tilapia, shrimp and salmon. Shrimp and tilapia can either can be fresh OR frozen. Salmon can be fresh or frozen but should be SKIN-ON, if possible. All seafoods can be domestically-reared or imported.

- You may collect more than one seafood sample from the same store if samples have different brands, or distributors, or sell by dates. Seafood can be purchased in bulk or by weight.

- Record the demographic information for each sample including store name and location, brand name, sell-by date, purchase date, lab processing date, and country of origin for each meat sample on the electronic log sheet. Please also record if the product was frozen, previously frozen, or fresh. If the package indicates whether the seafood was farm-raised or aquacultured, please indicate that as well.

- Please keep the following in mind: The monthly sampling and testing schedule is at your own discretion. Please keep in mind that fresh or previously frozen seafood expire more quickly than retail meats, and therefore will shorten the time to process, however frozen seafoods can be processed at later dates.

o The selection of fresh vs frozen and country of origin is at your own discretion. Please try to select as wide a variety as possible.

Sample Set-up:

Media should be brought to room temperature prior to inoculation for use on each day as needed below.

Do not open packages until ready to begin processing. **Fresh seafood samples should be processed within 96hrs after purchase**. Place intact packages of shrimp and salmon samples on a clean surface and aseptically open. **Preferably, do not thaw frozen samples before analysis.** If frozen sample must be tempered to obtain analytical portion, thaw below 45°C for <15 min with continuous agitation in thermostatically controlled water bath or thaw within 18hr at 2-5°C.
Aseptically remove shrimp or salmon samples with sterile instruments (e.g., tongs, gloves, or spoons).

***Vibrio* Species:**

Day 1: Aseptically weigh 25g of **shrimp and tilapia** separately into a sterile stomacher bag. Add 225ml of Alkaline Peptone Water (APW) and stomach for 2 min at 230 RPM or blend sample for 2 min.

Incubate this enrichment at 35°C for 24±2 hrs.

Day 2: Streak overnight enrichment onto TCBS agar incubate @35°C for 18-24hr. Repeat for each sample.

Day 3: Observe TCBS agar plates. If growth, pick green and yellow colonies. Pick one isolated colony of each color to a BAP. For each sample you can pick up to 1 to 2 colonies from TCBS agar.
Incubate each BAP @35°C for 24hr±2hr.

If no typical growth is observed, the sample is negative and can be discarded; complete the log sheet and select no for *Vibrio* spp.

Day 4: Examine each blood agar plate for purity and typical *Vibrio* colonies. **If you would like to do additional test you may do oxidase, gram stain, etc. It is not mandatory. FDA will identify all isolates sent to us.** If the growth is pure, swab the growth into Brucella broth with 15% glycerol mixture and freeze at -60 to -80°C. Repeat procedure for each BAP. Ship all isolates on dry ice to FDA-CVM. Laboratories should keep duplicates of strains within their culture collections.

Lactose Fermenters*, Enterococcus species* and *Aeromona*s

Day 1: Aseptically weigh 25g of shrimp, salmon and tilapia separately into a sterile stomacher bag. Add 225ml of Buffered Peptone Water (BPW) broth and stomach for 2 min at 230 RPM or blend sample for 2 min.

Incubate this enrichment at 35°C for 24±2 hrs.

Day 2:

Lactose Fermenters: Streak each sample to MacConkey agar and incubate @35°C for 24 hrs.

***Enterococcus species**:* Streak each sample to Enterococcosel agar and incubate @35°C for 24 hrs.

***Aeromonas*:** Streak each sample to CIN agar and incubate @ 25°C for 24-48hr.

Day 3:

Lactose Fermenters:

Examine each MAC plate for typical pink colonies. If no typical growth is observed on MAC agar plate, sample is negative and can be discarded, indicate results on log sheet for lactose fermenters. If typical growth is present, select one typical, well-isolated colony and streak for isolation onto a blood agar plate. Repeat procedure for each MAC plate. Incubate blood agar plate at 35°C for 24 hours.

***Enterococcus species*:**

Examine each Enterococcosel agar plate for typical *Enterococcus* colonies (surrounding medium black). If typical growth is present, select one typical, well-isolated colony and streak for isolation onto a BHI (or other non-blood containing) agar plate. Repeat procedure for each Enterococcosel agar plate. Incubate BHI plate(s) at 35°C for 24 hours

Aeromonas:

Examine each CIN agar for typical *Aeromonas* colonies. If typical growth is present, select one isolated. Pick deep red center with a margin, or "bull's eye" appearance. Streak one colony to BAP and incubate @35°C for 24hr±2hr. If no typical growth is observed on CIN agar plates, the sample is negative and can be discarded; indicate results on log sheet for *Aeromonas*.

Day 4:

Lactose Fermenters:. Examine each blood agar plate for pure colonies. If the growth is pure swab the growth into Brucella broth with 15% glycerol mixture and freeze at -60 to -80°C. Repeat procedure for each blood agar plate. Ship all isolates on dry ice to FDA-CVM. Laboratories should keep duplicates of strains within their culture collections

Enterococcus species: Examine each BHI agar plate for purity and typical enterococci colonies. If no typical growth is observed, sample is negative and can be discarded; complete the log sheet and select no for *Enterococcus*. If typical growth is observed, Gram stain the suspected colonies. If the Gram stain is atypical, sample is negative for enterococci and can be discarded; complete the log sheet and select no for *Enterococcus*. If Gram-positive cocci are observed, perform a catalase test. If catalase negative, confirm further with a PYR test. If catalase positive or PYR negative, plates may be discarded; complete the log sheet and select no for *Enterococcus*. If results produce catalase negative and PYR positive, record the isolate as *Enterococcus*. Repeat procedure for each BHI agar plate. Sub culture one well isolated colony from BHI to blood agar plate. Incubate at 35°C for 24 hours.

***Aeromonas*:** Examine each blood agar plate for purity and typical *Aeromonas* colonies. Pick one colony for oxidase test. **If it is oxidase positive, please keep for freezing. If it is oxidase negative, please discard.** If the growth is pure swab the growth into Brucella broth with 15% glycerol mixture and freeze at -60 to -80°C. Repeat procedure for each blood agar plate. Ship all isolates on dry ice to FDA-CVM. Laboratories should keep duplicates of strains within their culture collections.

Day 5:

***Enterococcus species**:* Examine each blood agar plate for purity and typical *Enterococcus* colonies. If the growth is pure swab the growth into Brucella broth with 15% glycerol mixture and freeze at -60 to -80°C. Repeat procedure for each BAP . Ship all isolates on dry ice to FDA-CVM. Laboratories should keep duplicates of strains within their culture collections.

Preparing Isolates for Shipment:
Please label each vial with NARMS isolate ID. **The NARMS isolate ID on the vial should match the NARMS isolate ID that you enter on the seafood log sheet.** Laboratories should keep duplicates of strains within their culture collections until notified by FDA-CVM that the duplicates may be discarded (isolates can be discarded once the NARMS report for the testing year has been published).

Isolate ID's should follow the following format:

Vibrio: Vibrio isolates should be labeled with the following format:
Year+State+Month+Source+SampleNumber-V+colony number (Example: 20SC08SH01-V1)

Lactose Fermenters: should be labeled with the following format:
Year+State+Month+Source+SampleNumber-LF (Example: 20SC08SH01-LF)

Enterococcus: Year+State+Month+Source+SampleNumber-E (Example: 20SC08SH01-E)

Aeromonas: Year+State+Month+Source+SampleNumber-A (Example: 20SC08SH01-A)

Source Key:

Shrimp: SH

Salmon: SA

Tilapia: TI

Packaging the Isolates:

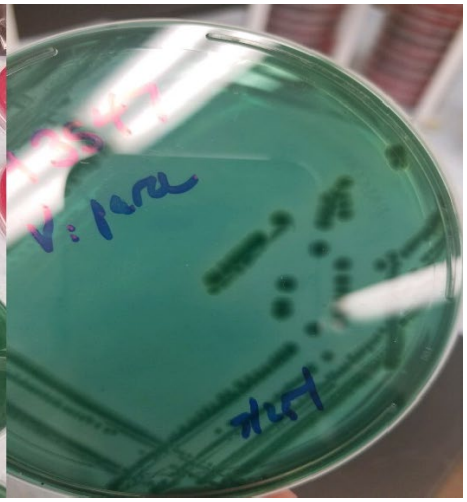
Ship all isolates in cryogenic vials with parafilm wrapped tops to keep tops from coming unscrewed. Cryogenic vials should be properly wrapped using bubble wrap, cotton, paper towels, etc. to ensure they do not break during shipping. Place cryogenic vials in a shipping container with plenty of dry ice placed in a box for shipping. Cryogenic vials should be shipped to FDA-CVM in accordance with current shipping of hazardous material guidelines. Prior to shipment of isolates to FDA-CVM, sites should e-mail a copy of the completed log sheets to NARMS seafood pilot study liaisons at FDA. The original log sheets or hard copies of electronic log sheets should be included with each isolate shipment to FDA-CVM. Each site should retain copies of log sheets for their records.

Shipping the Isolates:

Packages should be sent overnight. Please ship isolates so they will arrive at FDA-CVM by Thursday. If the isolates will not arrive by Thursday, please store them in your freezer and ship the following Monday. Shipments must occur monthly.

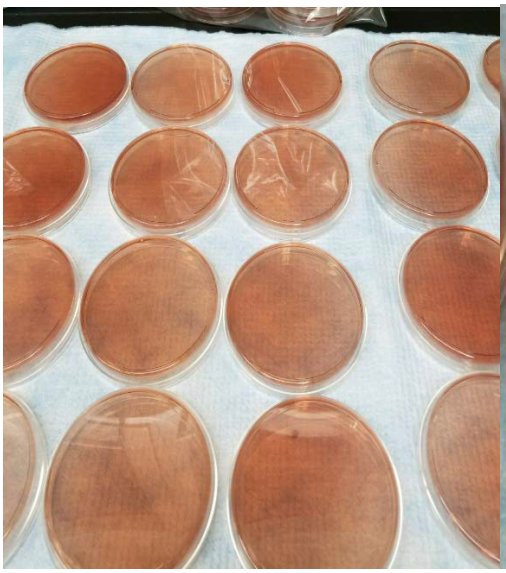
Pictures of Selective Agar Plates for Seafood Study:

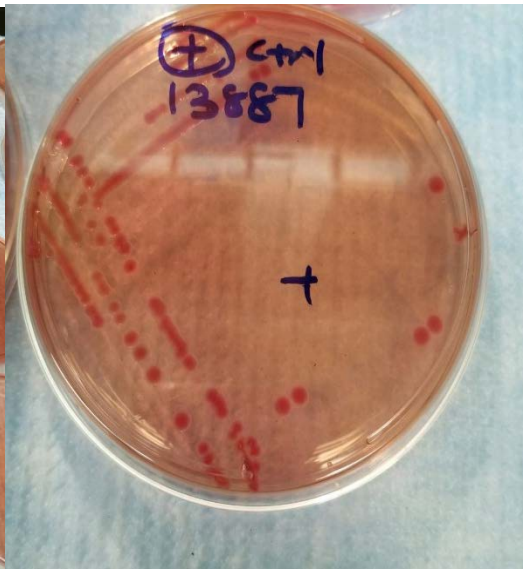
TCBS Agar- Use for Vibrio species



Unincoulated TCBS

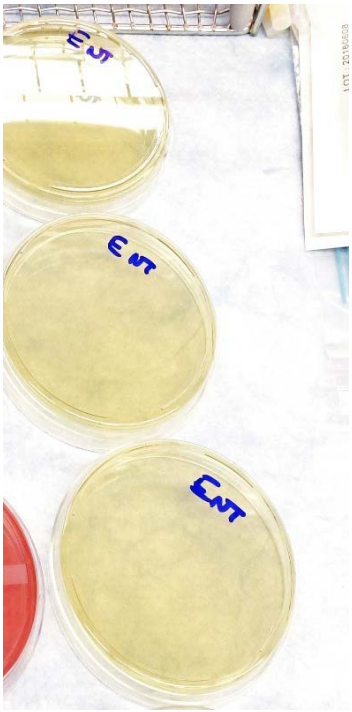
CIN AGAR- Use for Aeromonas

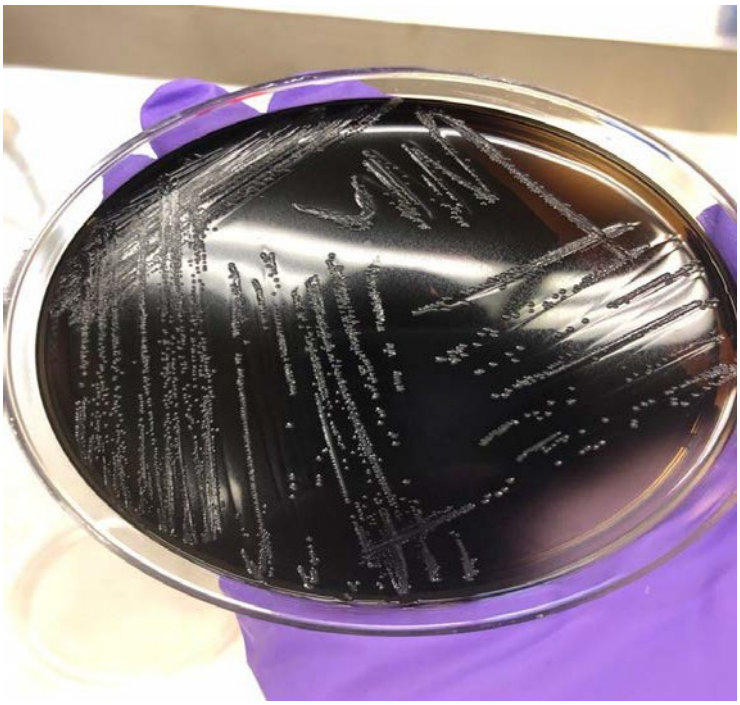




Uninoculated CIN Agar

Enterococcosel Agar- Use for isolating Enterococcus





Uninoculated Enterococcosel Agar

SEAFOOD PILOT FLOWCHART

Vibrio Species

DAY 1

Weigh 25g of Shrimp and Tilapia

Add 225ml of APW and stomach at 230RPM for 2 min or blend for 2 min.

Incubate @35°C for 24hr±2hr

DAY 2

Streak overnight enrichment onto TCBS agar incubate @35°C for 18-24hr.

DAY 3

Observe TCBS agar. If growth, pick green and yellow colonies. Pick one isolated colony of each color to a BAP. Incubate @35°C for 24hr±2hr

DAY 4

Examine each blood agar plate for purity. Sites should freeze each colony in Brucella broth with 15% glycerol mixture and ship all isolates on dry ice to FDA-CVM.

Lactose Fermenters, Aeromonas and Enterococcus

DAY 1

Weigh 25g of Shrimp, Salmon and Tilapia

Add 225ml of BPW and stomach at 230RPM for 2 min or blend for 2 min.

Incubate @35°C for 24hr±2hr

DAY 2

Lactose Fermenters:
Streak to MacConkey (MAC) agar and incubate *@35°C for 24hr.*

Aeromonas:
Streak to CIN agar and incubate @25°C for 24-48hr.

Enterococcus:
Streak to Enterococcosel Agar (EN) @35°C for 24hr.

DAY 3

Lactose Fermenters:
Pick one isolated colony. Lactose positive (Pink) from Mac plate to BAP agar @35°C for 24hr

Aeromonas:
Pick one isolated colony from CIN agar. Pick deep red center with a transparent margin, or"bull's-eye" appearance and streak to BAP agar for @35°C for 24hr±2hr.

Enterococcus:
Pick surrounding medium black colony and streak to BHI Agar. Incubate @35°C for 24hr±2hr.

DAY 4

Lactose Fermenter:
Examine each blood agar plate for purity. Sites should freeze each colony in Brucella broth with 15% glycerol mixture and ship all isolates on dry ice to FDA-CVM.

Aeromonas:
Examine each blood agar plate. Perform oxidase test on one isolated colony. If oxidase positive freeze down in Brucella broth with 15% glycerol mixture and ship all isolates on dry ice to FDA-CVM. If oxidase negative discard.

Enterococcus:
Examine the BHI agar plates. See detailed protocol for further testing of Enterococcus isolates.

DAY 5

Enterococcus:
Examine each blood agar plate for purity. Sites should freeze each colony in Brucella broth with 15% glycerol mixture and ship all isolates on dry ice to FDA-CVM.