

APPENDIX T
OHIO WATER MICROBIOLOGY LABORATORY
Verification of enterococci colonies from mEI (US EPA Method 1600)

Overview:

The following is a summary of the verification procedure for enterococci colonies on mEI from USEPA Method 1600 (U.S. Environmental Protection Agency, 2006). The reader is encouraged to read the full method, available at

<http://www.epa.gov/waterscience/methods/method/biological/>

Colonies ≥ 0.5 mm in diameter of any color having a blue halo after incubation on mEI agar are considered to be “typical” enterococci colonies (USEPA, 2006). Verification is recommended as a means of quality control and is suggested when questions arise regarding positive colonies (i.e., pinpoint colonies). Isolated colonies are used to inoculate a nutrient broth and agar. Growth from the broth is used to inoculate additional mediums to determine growth capabilities in salt and at higher temperatures, as well as, the ability of the organism to hydrolyze esculin. A Gram stain is done to ensure the organism is a gram positive cocci.

Media and reagents:

Brain Heart Infusion Broth (BHIB)

Composition:

Calf brains, infusion from 200.0 g	7.7 g
Beef heart, infusion from 250.0 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Dextrose	2.0 g
Reagent grade water	1.0 L

Heat and stir to dissolve. Dispense into 10-mL volumes in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2 . Store at 4°C.

Alternately, commercially available, dehydrated broth (bacto) can be purchased (BD 237400).

Brain Heart Infusion Broth with 6.5% NaCl (BHIB w/6.5% NaCl)

Composition is the same as BHIB above, but with addition of NaCl:

BHIB	1 L
NaCl	60.0 g

Heat and stir to dissolve. Dispense into 10-mL volumes in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2 . Store at 4°C.

Brain Heart Infusion Agar (BHIA)

Composition is the same as BHIB above, but with the addition of agar:

BHIB	1 L
Agar	15.0 g

Heat and stir to dissolve. Dispense Autoclave at 121°C (15 PSI) for 15 minutes. Slant until solid. Final pH should be 7.4 ± 0.2 . Store at 4°C.

Bile Esculin Agar (BEA)

Composition:

Beef Extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Oxgall	20.0 g
Esculin	1.0 g
Ferric citrate	0.5 g
Bacto agar	14.0 g
Reagent grade water	1.0 L

Heat and stir to dissolve. Boil for 1 minute to dissolve completely. Dispense into 10-mL volumes in screw cap tubes for slants or larger volumes in screw cap bottles for plates, and autoclave at 121°C (15 PSI) for 15 minutes. Overheating may cause darkening of the medium. Cool in 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.8 ± 0.2 . Store at 4°C. Store in refrigerator.

Alternately, commercially available, dehydrated agar can be purchased (BD 299068).

Method:

1. Identify isolated colonies for verification and start the lab bench sheet. A blank should be included for each step and media type.
2. Pick isolated colonies and control cultures (Table 1) and inoculate a 10-mL tube of BHIB and a slant of BHIA.
 - a. Incubate the BHIB at 35°C for 24 hours.
 - b. Incubate the BHIA slant at 35°C for 48 hours.
3. After 24-hour incubation in BHIB, transfer a loopful of growth to BEA slant, BHIB, and BHIB w/6.5% NaCl.
 - a. Incubate BEA and BHIB w/6.5% NaCl at 35°C for 48 hours.
 - b. Incubate the BHIB at 45°C for 48 hours.
4. After 48-hour incubation on BHIA slant, perform a gram stain.
5. Observe all media for growth. Enterococci positive verification:
 - a. Growth in BHIB w/6.5% NaCl.
 - b. Growth in BHIB incubated at 45°C.
 - c. Black or brown precipitate on BEA (hydrolysis of esculin).
 - d. Gram positive cocci.

Table 1. Control cultures for verification media.

Medium	Positive control	Negative Control
Bile esculin agar (BEA)	<i>E. faecalis</i> (ATCC 19433)	<i>E. coli</i>
Brain heart infusion broth (BHIB) with 6.5% NaCl	<i>E. faecalis</i> (ATCC 19433)	<i>E. coli</i>
Brain heart infusion broth (BHIB) incubated at 45°C	<i>E. faecalis</i> (ATCC 19433)	<i>E. coli</i>

Reference: U.S. Environmental Protection Agency, 2006, Method 1600: Enterococci in water by membrane filtration using membrane-Enterococcus Indoxyl- β -D-Glucoside: Washington D.C., EPA 821-R-06-009, 24 p.



Enterococci Verification

Analyzed by (initials): _____ Analysis Start Date: _____
 BHIB prep date: _____ BHIA prep date: _____
 BHIB w/6.5% NaCl prep date: _____ BEA prep date: _____
 Gram stain kit lot #s:
 Crystal violet: _____ Iodine solution: _____
 Decolorizer: _____ Safranin: _____

Day 1. Time BHIB and BHIA in 35°: _____

Day 2. Secondary inoculation:

- a. Time BHIB in 45°: _____
- b. Time BEA and BHIB w/6.5% NaCl in 35°C: _____

Day 3. Gram Stain (from BHIA slant)

- a. Slide + control results: _____
- b. Slide - control results: _____

Day 4. Observe secondary media for growth

- a. Time BHIB (45°C) out: _____
- b. Time BEA out: _____
- c. Time BHIB w/6.5% NaCl out: _____

Results

Isolate ID	Source	Growth in BHIB at 45°C	Growth in BHIB w/6.5% NaCl	Precipitate on BEA	Gram Stain description