

Testing Methodology for Detection and Isolation of *Salmonella* spp. from Agricultural Soils and Sediment

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I. Introduction

The method described here is for testing agricultural soils and sediment samples for *Salmonella* spp. Fresh produce can be contaminated through various routes at the pre- and post-harvest stages. Irrigation water and agricultural soils are the primary reservoirs and transmission routes of human pathogens at the preharvest stage due to the long-term survival of pathogens in these environmental niches (1, 2). Soils used to grow produce may serve as reservoirs of foodborne pathogens, particularly soils treated with biological soil amendments of animal origin (BSAAO) (3). On-site farm investigations serve as a critical component in both foodborne illness traceback efforts and root cause analysis (4, 5).

A. Equipment and materials

1. Electronic top-loading balance capable of weighing a minimum of 25 ± 0.1 g (500 g capability recommended)
2. Sterile, Whirlpak-type bags with filter, 7.5 in x 12 in (19 cm x 30.5 cm)
3. Incubator, $35 \pm 2^\circ$ C
4. Water bath, circulating, thermostatically controlled, $42.0 \pm 0.2^\circ$ C and $43.0 \pm 0.2^\circ$ C
5. Sterile serological pipets
6. Sterile disposable loops (10 μ l) and inoculating needles
7. Sterile spatulas or scoops
8. Transport container with gel packs

B. Media and Reagents

1. Modified buffered peptone water (mBPW; [M192b](#))
2. Tetrathionate (TT, [M145](#)) broth
3. Rappaport-Vassiliadis (RV, [M132](#)) medium
4. Hektoen enteric novobiocin (HE+N; See section G) agar
5. Xylose lysine Tergitol 4 (XLT4; See section G) agar

C. Shipment

All samples should be placed in an insulated transport container with frozen gel packs to keep the samples cool. Shipment must contain appropriate collection reports for the laboratory.

D. Preparation of test portions

Mix the soil/sediment sample thoroughly by hand. Weigh a 25 g sample using sterile spatulas or scoops into the whirlpak-type bag. Aseptically add 225 ml of modified buffered peptone water (mBPW) to the bag with sample. Close the bag and hand-massage for 2 minutes to ensure proper mixing. Incubate at $35 \pm 2^\circ\text{C}$ for 24 ± 2 h.

E. Isolation

1. After incubation, shake the bag containing the soil/sediment sample and mBPW.
2. Transfer 1 ml of the incubated pre-enrichment into 10 ml of tetrathionate (TT) broth.
3. Transfer 0.1 ml of the incubated pre-enrichment to 10 ml of Rappaport-Vassiliadis (RV) medium.
4. Incubate the TT broth at $43.0 \pm 0.2^\circ\text{C}$ in a circulating water bath and the RV medium at $42.0 \pm 0.2^\circ\text{C}$ (water bath) for 24 ± 2 h.
5. After incubation, mix (vortex, if tube) and streak a loopful ($10\ \mu\text{l}$) of incubated TT broth on Hektoen enteric novobiocin (HE + N) agar and xylose lysine tergitol 4 (XLT4) agar plates.
6. Repeat this procedure with the RV medium.
7. Incubate all plates for 24 ± 2 h at $35 \pm 2^\circ\text{C}$.
8. Examine plates for colonies with morphology typical for *Salmonella*. Typical *Salmonella* colonies are as follows:
 - a. HE+N agar: blue-green or green with black centers, or entirely black.
 - b. XLT4 agar: black or black-centered colonies with a yellow or red/pink periphery after 18-24 hours of incubation.
9. In the absence of typical *Salmonella* colonies, examine plates for colonies with morphology atypical for *Salmonella*. Atypical *Salmonella* colonies are as follows:
 - a. HE+N agar: blue-green or green without black centers; or yellow, salmon, or orange colonies with or without black centers.
 - b. XLT4 agar: red/pink colonies without black centers; or yellow colonies with or without black centers.
10. Pick 5 or more typical (or atypical) colonies from each selective agar plate after ± 2 h incubation. Using a sterile inoculating needle, lightly touch the very center of the colony to be picked and streak each pick onto both XLT4 and HE+N to confirm purity and colony morphology. Incubate all plates for 24 ± 2 h at $35 \pm 2^\circ\text{C}$.
11. Examine each pair of (HE+N and XLT4) plates from step 10. Each pair of plates should be evaluated collectively for purity and morphology. Further, only one colony from each pair should proceed with confirmation (F.).

F. Confirmation procedures

1. The purified colonies are confirmed using the methods described in BAM Chapter 5, Section F.8.

Optional picked colonies can be screened with TSI and LIA slants following BAM Chapter 5, Section E.8. – E.10. before proceeding to F.8.

G. Media preparations

1. Hektoen enteric novobiocin (HE+N) agar

Peptone	12.0 g
Yeast extract	3.0 g
Bile salts No. 3	9.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
NaCl	5.0 g
Sodium thiosulfate	5.0 g
Ferric ammonium citrate	1.5 g
Bromthymol blue	0.065 g
Acid fuchsin	0.1 g
Agar	14.0 g
Distilled water	1 liter

- Dissolve ingredients in distilled water. Heat boiling with frequent agitation to completely dissolve the powder. Boil no longer than 1 min. Do not overheat. Do not autoclave.
- Cool to 50-55°C in a water bath. Before pouring, add 1 mL of 5 mg/mL novobiocin stock solution to 1 liter of basal medium to achieve a final concentration of 5 µg/mL and swirl thoroughly to mix.
- Dispense 20 ml portions into sterile 15 × 100 mm petri dishes. Let dry 2 h with lids partially removed.
- Final pH, 7.5 ± 0.2.
- Store the plates under refrigeration (4 ± 2°C) for up to 30 days.

Preparation of novobiocin salt stock solution (5 mg/mL)

- Mix 0.25g of novobiocin salt thoroughly in 50 ml of distilled water until completely dissolved.
- Filter-sterilize the solution using a 0.22 µm filter.

2. Xylose lysine Tergitol 4 (XLT4) agar

Proteose peptone	1.6 g
Yeast extract	3.0 g
L-lysine	5.0 g
Xylose	3.75 g

Lactose	7.5 g
Saccharose	7.5 g
Ferric ammonium citrate	0.8 g
Sodium thiosulfate	6.8 g
Sodium chloride	5.0 g
Agar	18.0 g
Phenol red	0.08 g
Distilled water	1 liter

- Dissolve ingredients in distilled water
- Add 4.6 ml XLT4 agar supplement (Tergitol 4)
- Heat boiling with frequent agitation to completely dissolve the powder. Boil no longer than 1 min. Do not overheat. Do not autoclave.
- Cool to 50-55° C in a water bath and dispense 20 ml portions into sterile 15 × 100 mm petri plates. Let dry 2 h with lids partially removed.
- Final pH, 7.4 ± 0.2.
- Store the plates under refrigeration (4 ± 2°C) for up to 30 days.

H. References

1. Bell RL, Kase JA, Harrison LM, Balan KV, Babu U, Chen Y, Macarisin D, Kwon HJ, Zheng J, Stevens EL, Meng J, Brown EW. The Persistence of Bacterial Pathogens in Surface Water and Its Impact on Global Food Safety. *Pathogens*. 2021 Oct 27;10(11):1391. doi: 10.3390/pathogens10111391.
2. Erickson MC, Habteselassie MY, Liao J, Webb CC, Mantripragada V, Davey LE, Doyle MP. Examination of factors for use as potential predictors of human enteric pathogen survival in soil. *Journal of Applied Microbiology*. 2014 Feb 1;116(2):335-49.
3. Olaimat A.N., Holley R.A. Factors influencing the microbial safety of fresh produce: A review. *Food Microbiol*. 2012;32:1–19. doi: 10.1016/j.fm.2012.04.016.
4. FDA 2022: <https://www.fda.gov/food/outbreaks-foodborne-illness/factors-potentially-contributing-contamination-packaged-leafy-greens-implicated-outbreak-salmonella>
5. FDA 2023: <https://www.fda.gov/food/outbreaks-foodborne-illness/factors-potentially-contributing-contamination-cantaloupe-implicated-outbreak-salmonella-typhimurium>