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# *Salmonella* spp. detection and isolation

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MATERIALS TEXT

1. 500-mL Whirl-pak Write-on sampling bags

2. Modified Buffered Peptone Water:

| | |
|---|---|
| Pancreatic Digested of Gelatin | 5.0 g |
| Beef Extracts | 5.0 g |
| Sodium Chloride | 5.0 g |
| Disodium Phosphate (Na2HPO4) | 7.0 g |
| Monopotassium Phosphate (KH2PO4) | 3.0 g |
| Distilled water | 1000 mL |

3. Buffered Peptone Water:

| | |
|---|---|
| Enzymatic Digested of Casein | 10.0 g |
| Sodium Chloride | 5.0 g |
| Disodium Phosphate (Na2HPO4) | 9.0 g |
| Monopotassium Phosphate (KH2PO4) | 1.5 g |
| Distilled water | 1000 mL |

4. Rappaport-Vassiliadis-Soy broth (Difco)

5. Tetrathionate broth (Bioxon)

6. InstaGene Matrix (BIORAD. Cat. No. 732-6030)

7. 5x FIREPol® Master Mix

8. invAF1 (26 bp). CTGCTTTCTCTACTTAACAGTGCTCG (100 nM scale, desalted)

9. invAF2 (22 bp). CGCATCAATAATACCGGCCTTC (100 nM scale, desalted)

10. Nuclease-free molecular grade water

11. Biotechnology-grade agarose (NORGEN)

12. PCR loading buffer Orange G 6x

13. GelRed 10,000X

14. Ready-to-load DNA ladder 100 bp (Thermofisher Scientific)

15. Buffer tris-borate-EDTA (TBE) 10x

16. 70% alcohol

17. XLT4 medium and supplement (Difco)

18. Salmonella CHROMAgar medium (ready to use plates)

19. Soy tripticase agar (TSA, BIOXON)

20. Brain-heart-infusion broth (BHI, BIOXON)

21. Glycerol (SILVERQUIM)

22. Mineral oil (COSMOPOLITA)

## 1.1 Pre-enrichment of surface water samples 1d

1 Close to a gas burner, open the plastic bag containing the modified Moore Swab (MMS) and aseptically take out the



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cheesecloth from inside the MMS.

2 Place the cheesecloth inside a 500-mL Whirl-pak bag, previously identified with sample name.

3 Pour 200 mL of modified buffered peptone water (mBPW) into the Whirl-pak bag, close the bag and carefully squeeze and massage the cheesecloth for 2 min to ensure contact of the cheesecloth with the mBPW.

4 Place the bags in a shaking incubator at 36-37 ºC / 18-20 h / 150 rpm.

1.2 Pre-enrichment of meat and lymph node samples 1d

5 **Lymph nodes (LN):**

Weight LN and place them in boiling water for 5 s to sterilize their surface. Afterwards, prepare 1:10 dilutions of LN samples (i. e. 8 g LN in 72 mL BPW or 25 g LN in 225 mL BPW). Use half of BPW volume to grind LN samples for approximately 30 s in a sterile Oster blender. Transfer the ground sample to a Stomacher bag, previously identified with sample name. Use the remaining volume of BPW to rinse the blender cup several times until all the ground sample is transferred to the Stomacher bag. Homogenize the sample in the Stomacher machine for 1 min. Let it stand for 2 h at room temperature before incubating at 42 ºC / 18-20 h.

6 **Meat cuts, minced meat or ground meat:**

Aseptically weight 25 g of meat and grind it for 30 s in a sterile Oster blender. Aseptically transfer the ground meat to a Stomacher bag, add 225 mL of BPW and homogenize for 1 min. If dealing with ground meat, skip the grinding step. Let the homogenized sample stand for 2 h at room temperature before incubating at 36-37 ºC / 18-20 h.

2. Selective enrichment 1d

7 Using sterile tips and automated pipettes, take 100 µL of the pre-enriched sample and place in an assay tube containing 10 mL of Rappaport-Vassiliadis-Soy (RVS) broth. Likewise, take 1 mL of the pre-enriched sample and place it in an assay tube containing 9 mL of tetrathionate (TT) broth.



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8 Close the caps of the tubes and incubate RVS and TT broths at 43 ºC and 150 rpm for 18-24 h.

## 3. PCR screening of selective enriched samples 1d

9 **DNA extraction from selective enriched samples**

Take 500 µL aliquots of each selective enrichment broth (RVS and TT) and mix them in a 1.5-mL Eppendorf tube.

10 Centrifuge at 11,000 rpm for 1 min and discard the supernatant.

11 Mix the Instagene reagent at half speed in a magnetic stirrer. Then, use a 1000-µL pippete tip to add 200 µL of InstaGene to the tube.

12 Place the tube in a termoblock at 56 ºC for 15 min. Afterwards, vortex the tube at full speed for about 10 s.

13 Place the tube back to the termoblock at 100 ºC for 8 min. Vortex again at full speed for 10 s.

14 Centrifuge at 11,000 rpm for 3 min and take 20-50 µL of the supernatant to run the PCR test.

## 4. *Salmonella* detection by PCR targeting the *invA* gene (amplification of a 284 bp fragment) 8h

15 In the pre-PCR cabinet, prepare enough master mix to run the number of required PCR reactions. Use the following table as a reference:

**The PCR test is standardized for a total volume of reaction = 10 µL.***

| Reagent | µL / 1 reaction | µL / 10 reactions | µL / 20 reactions |
|---|---|---|---|
| 5X Firepol Master Mix | 5.0 | 50.0 | 100.0 |
| dNTP-F (1) | 0.2 | 2.0 | 4.0 |
| dNTP-R1 (1) | 0.2 | 2.0 | 4.0 |
| NF-water (2) | 2.1 | 21.0 | 42.0 |
| Master mix volume | 7.5 | 75.0 | 150.0 |
| DNA sample (3) | 2.5 | 2.5 | 2.5 |

(1) Forward primer: gtgaaattatcgccacgttcgggcaa. Reverse primer: tcatcgcaccgtcaaaggaacc. These primers are based on methods of Rahn et al. 1992. doi: 10.1016/0890-8508(92)90002-f
(2) NF-water: nuclease-free molecular grade water.
(3) **DNA sample is added last and in a different PCR cabinet.**



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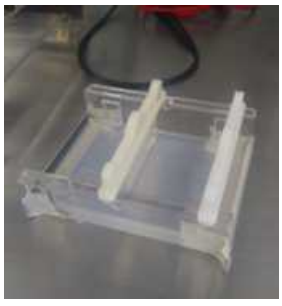
*Always include a blank (7.5 µL master mix + 2.5 µL NF-water) in every run, as well as a DNA extraction from our reference *S. enterica* subsp. *enterica* ser. Typhimurium strain as a positive control.

Run the PCR reactions in the thermocycler, programmed as follows:

| Step | Conditions | No. of cycles |
|---|---|---|
| Initial denature | 94 ºC / 3 min | 1 |
| Denature | 95 ºC / 45 s | 35 |
| Annealing | 62 ºC / 30 s | 35 |
| Extension | 72 ºC / 45 s | 35 |
| Final extension | 72 ºC / 5 min | 1 |
| Hold | 4 ºC | -- |

## 5. Visualization of PCR-amplified fragments 4h

16 Prepare 2% agarose gel. For 35 mL of agarose gel, mix 0.7 g of agarosa and 35 mL of buffer tris-borate-EDTA (TBE). Heat in microwave until complete disolution of agarose. Let it cool down (65 ºC approx.), pour it in the mold and place the comb to make wells. Let it cool down to room temperature and take it out of the mold when ready to run the PCR amplification products.



17 Prepare enough loading buffer for the number of samples to run (1 µL of loading buffer per sample).

Perform this procedure in the pre-PCR cabinet. Use a 1.5-mL vial to mix 1 mL of loading buffer 6X with 1 µL of red gel. Shake well and keep refrigerated and protected from light until used.



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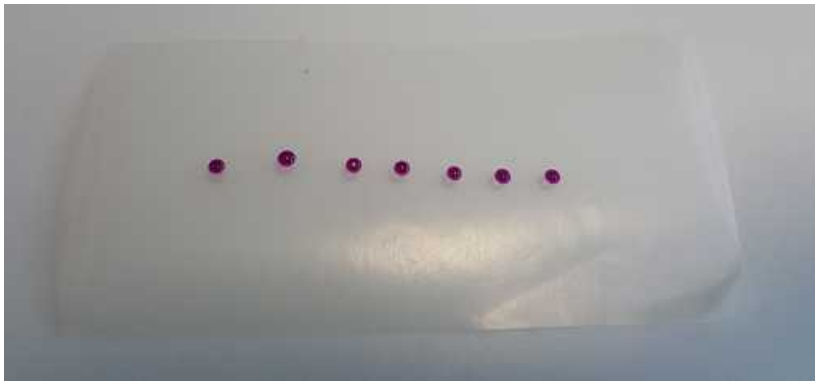
18 Place the 2% agarose gel in the electrophoresis chamber. Fill the electrophoresis chamber with enough 1X buffer TBE until it slightly goes over the agarose gel. On the benchtop, use a parafilm fragment and pour 1 µL aliquots of loading buffer across the parafilm.



Take 5 µL of each sample to run. After that, set the pipette volume to 8-10 µL and carefully absorb the loading buffer with the aid of the pipette, mixing gently several times. Load the sample in the corresponding well of the agarose gel. Change pipette tips between samples, blank and positive control. **DO NOT USE THIS PROCEDURE to load DNA ladder since it is ready to use. The DNA ladder (6 µL) is loaded directly into the corresponding well.**

19 Close the electrophoresis chamber and connect it to its power source. The wells on the gel should always be on the negative side of the chamber. Set voltage to 80 V and running time to 50 min and start the run.

20 Once the electrophoresis is finished, turn off the power supply and get the gel out of the chamber. Tilt the gel tray over the chamber to remove the excess TBE buffer from the gel. Bring the gel to the photo-documentation system.

21 Open the photo-documentation device and clean it with 70% alcohol before placing the gel to be documented. Use a disposable paper towel to dry any excess of alcohol left on the surface.

22 Place the gel on the clean surface of the photo-documentation system, close the cabinet and turn it on. Open the gel documentation software in the connected computer. Use the program to digitalize the gel image. Edit the image to identify each of the lanes (DNA ladder, blank, positive control, samples) and save it. The file name of the image is composed as follows: project initials_date_number_of_samples_in_that_run. For example, if documenting samples 1 to 20 of "Project1" on November 18, 2020, the file name must be: project1_111820_1to20.jpeg



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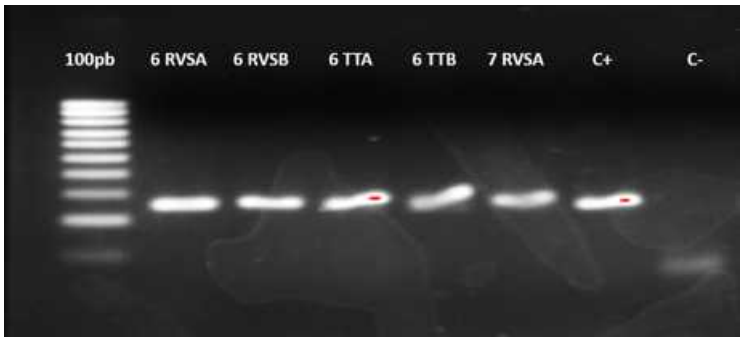
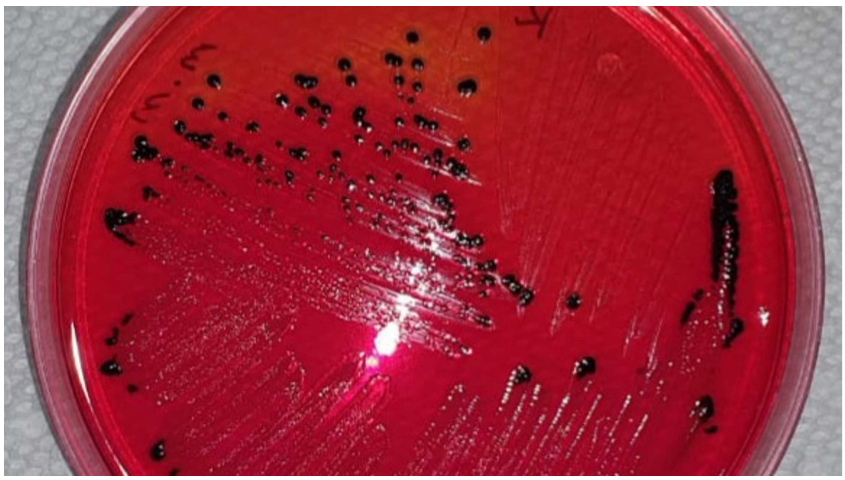



## 6. Selective plating, isolation and final identity confirmation 3d

23 Selective enriched samples that were negative in the PCR test are discarded. For positive samples, take 100 µL from each selective enrichment broth and streak it in duplicates in XLT-4 and Salmonella CHROMagar plates.

24 Incubate plates at 35 ± 1 ºC/ 24 ± 2 h. After incubation, check for colonies with typical *Salmonella* morphology.



*Salmonella* morphology in XLT-4 agar: black colonies, with a convex surface and regular rounded shape.



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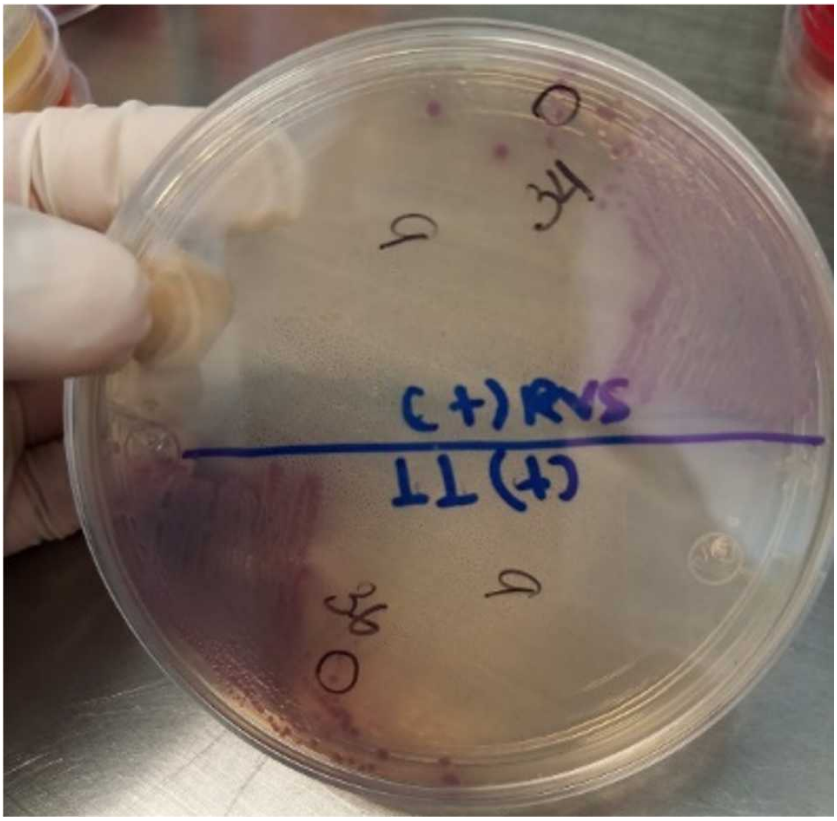


*Salmonella* morphology in Salmonella CHROMagar: mauve colonies, with a regular rounded shape.

25 Pick at least 3 typical isolated colonies from each selective agar for final PCR identity confirmation. Mix each isolated colony in 1 mL of molecular grade water. Afterwards, conduct steps 8-12 of section 3, as well as all steps from sections 4 and 5 of this protocol.

26 **Only for samples with positive results in final PCR confirmation:**

Pick some growth from the same colonies used for final PCR confirmation and streak it into TSA agar plates. Incubate at 37 ºC / 24 h. Afterwards, pick isolated colonies from the TSA agar plate and preserve pure isolates, as described below.

## 7. Preservation of pure isolates 2d

27 **Deep frozen storage**

With a sterilized inoculation loop, pick isolated colonies from TSA agar plates and transfer them to 5-mL Falcon tubes containing 2 mL of sterile BHI broth. Place the tubes in a shaking incubator at 37 ºC for 24 h and 150 rpm. After incubation, add 0.4 mL of sterile glycerol to each tube, mix well and store at - 80 ºC.



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28 **Room temperature storage**

With a sterilized inoculation loop, pick isolated colonies from TSA agar plates and inoculate them into TSA slants. To do that, first stab the center of the medium on the lowest part of the slant. Then, take the loop out and streak on the surface of the agar slant, moving outwards. Fill up to 3/4 of the streaked tubes with mineral oil. Close the tubes and seal the caps with parafilm. Keep pure isolates at room temperature.

Disposal of wastes

29 Discard all generated residues according to current procedure to handle hazardous wastes.



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