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Isolation and qPCR for S. Typhi detection from River Water Samples

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We use this protocol and it's working

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Abstract

This protocol outlines a validated methodology for water surveillance of *Salmonella enterica* serovar Typhi (*S*. Typhi) through optimised field sampling, selective culture, and confirmatory real-time PCR. It includes detailed steps for grab sample and Moore swab collection, laboratory processing, DNA extraction, and molecular confirmation.

Guidelines

Current guidelines recommended by the Typhoid Environmental Surveillance Consortium can be found at **https://www.typhoides.org/** under protocols.

Materials

- Sterile 500 mL or 1 L Nalgene bottles
- Moore swabs (6" x 48", or 15 cm x 120 cm, sterile gauze, folded 8-ply and tied with fishing line)
- Sterile Whirl-Pak bags
- GPS-enabled tablet or field notebook
- Multiprobe water meter (turbidity, pH, temperature, conductivity)
- PPE: gloves, masks, boots
- Cooler box with ice packs (2–8°C transport)
- 70% ethanol for hand and surface sanitation
- Sample ID labels and collection forms (paper/digital)

	A	B
	Distilled Water	1,000 mL
	Ox Bile (Ref. NCM0240A; Neogen, Manchester, UK)	20 g
	Dextrose (Ref. NCM0241A; Neogen)	5 g
	Peptone from gelatin, pancreatic digest (Ref. 70176, Merck, Hertfordshire, UK)	10 g
	Sodium phosphate dibasic dihydrate (Ref. 71643, Merck)	8 g
	Potassium dihydrogen phosphate (Ref. NIST200B, Merck)	2 g

Table 3: Recipe for Bile- Broth:

	A	B
	Distilled Water	1,000 mL
	Selenite Broth Base (Ref. CM0395, Oxoid, Basingstoke, UK)	38 g
	Sodium Biselenite (Ref. LP0121, Oxoid)	8 g

Table 4: Recipe for Double Strength Selenite F broth

Step 1: Site Selection and Metadata Logging

1 Use GIS-informed mapping to identify candidate water bodies (streams, rivers, drainage).

2 Record GPS coordinates and environmental context for each site.

3 Assess for accessibility and safety; log human or animal activity and environmental conditions.

4 Complete the site metadata form with:

- Date/time
- Weather conditions
- Recent rainfall
- Visible contamination
- Sample type(s) deployed
- Field team ID

Step 2: Grab Sample Collection

5 Don appropriate PPE.

6 Submerge sterile Nalgene bottle ~30 cm below the surface, upstream of your body position.

7 Collect 1 litre of water.

8 Seal bottle.

9 Change gloves and clean surfaces with 80% ethanol.

10 Affix sample label.

11 Log sample ID, time, temperature, turbidity, pH, and conductivity.

Step 3: Moore Swab Preparation and Deployment

12 Prepare swab by folding sterile gauze in a pleated/accordion fashion.

13 Tie center securely with sterile fishing line (1–2 m retrieval length).

14 Further instructions can be found at:
Construction of a Moore's Swap - Typhoid ES Consortium
or, **Sikorski and Levine, 2020**
- See Protocol References at the end of this document

15 Place swab into mid-stream using line secured to a stable structure (e.g., bridge, tree).

16 Deploy for 48–72 hours.

17 Retrieve place directly into sterile Whirl-Pak bag.

18 Change gloves and clean surfaces with 80% ethanol.

19 Label bag and log:

- Deployment duration
- Retrieval time
- Any swab loss events

Step 4: Sample Transport and Storage

20 Place all samples in cooler boxes at 2–8°C.

21 Transport to lab within 3 hours ideally, 6–8 hours maximum.

22 If delay to processing is unavoidable, store at 4°C and process within 24 hours.

23 Record condition on arrival and any temperature deviations.

Step 5: Pre-enrichment – Primary Broth Incubation

24 For water: filter as much of the sample as possible through 0.45 µm membrane.

- If filters block, change the filter up to 5 membranes
- Stop filtering after 1 hour per sample
- Any water that was not filtered, record volume remaining

25 Place samples into culture media:

1. Resuspend filter in 10 mL 2% bile$^-$ broth
2. For Moore swabs: directly submerge whole swab into 250 mL 2% bile$^-$ broth.

26 Incubate at 37 ± 2 °C for 18–24 h.

Step 6: Selective Enrichment – Secondary Broth

27 Aseptically transfer 5 mL of incubated bile$^-$ broth into 5 mL of double strength Selenite F broth.

28 Incubate at 41 ± 2 °C for 12–18 h (static incubation).

29 Label all broths with corresponding sample ID and time.

Step 7: Selective Plating on mCASE Agar

30 Plate the culture media onto modified Chromogenic Agar *Salmonella* esterase (mCASE) media:

1. Streak for single colonies
2. Spread plate at 1:10 dilution
3. Spread plate at 1:100 dilution

31 Incubate at 37°C for 18–24 h.

32 Observe for blue-green colonies (indicative of *S*. Typhi).

33 Pick presumptive colonies for molecular confirmation.

Step 8: DNA Extraction

34 DNA extraction was performed on isolates using the boilate, or thermal lysis, method

35 Add 500 uL of nuclease free water to a 1.5 mL microcentrifuge tube

36 Pick 1 to 2 colonies and emulsify into the tube

37 Heat at 96 °C for 10 minutes

38 Centrifuge at full speed for 5 seconds to remove condensation

Step 9: Real-Time PCR Confirmation (Triplex Assay)

39 Prepare reaction mix (25 µL total volume):

	A	B	C	D	E	F
	Reagent	Working Concentration	Volume of Working Conc	Final Concentration	Seqeunce of Primer/Probe	References
	Takyon Blue 2x Master Mix with ROX	2x	12.5 µL	1x	-	
	ttr Forward Primer	20 µM	0.25 µL	200 nM	CTCACCAGGAGATTACAACATGG	(Hopkins et al., 2009)
	ttr Reverse Primer	20 µM	0.25 µL	200 nM	AGCTCAGACCAAAAGTGACCATC	(Hopkins et al., 2009)
	ttr Probe (FAM - BHQ1)	5 µM	0.5 µL	100 nM	CACCGACGGCGAGACCGACTTT	(Hopkins et al., 2009)
	tviB Forward Primer	20 µM	0.5 µL	400 nM	TGTGGTAAAGGAACTCGGTAAA	(Nair et al., 2019)
	tviB Reverse Primer	20 µM	0.5 µL	400 nM	GACTTCCGATACCGGGATAATG	(Nair et al., 2019)
	tviB Probe (TET-BHQ1)	5 µM	1 µL	200 nM	TGGATGCCGAAGAGGTAAGACGAGA	(Nair et al., 2019)
	staG Forward Primer	20 µM	0.5 µL	400 nM	CGCGAAGTCAGAGTCGACATAG	(Nga et al., 2010)
	staG Reverse Primer	20 µM	0.5 µL	400 nM	AAGACCTCAACGCCGATCAC	(Nga et al., 2010)
	staG Probe (Cy5 - BHQ2)	5 µM	1 µL	200 nM	CATTTGTTCTGGAGCAGGCTGACGG	(Nga et al., 2010)
	Nuclease Free Water	-	2.5 µL	-	-	
	Total Working Volume	-	20 µL	-	-	
	DNA Template Volume	-	5 µL	-	-	

Table 1: qPCR Master Mix for *S.* Typhi detection

40 Cycling Conditions:

	A	B	C	D
	Stage	Temperature	Time	Cycles
	Hold Stage	95 °C	5 Minutes	1
	Denaturation	95 °C	30 Seconds	40
	Annealing	60 °C	30 Seconds	
	Extension	72 °C	10 Seconds	
	Ramp up Speed	2.05 °C/s		
	Reaction Volume	25 µL		

Table 2: Thermocycling Conditions

Step 10: Data Analysis and Interpretation

41 Positive sample = amplification of all three targets (ttr, tviB, staG) with Ct < 38.

42 Include controls:

1. NTC
2. qPCR Negative
3. Positive control

43 Record Ct values, amplification plots, and annotate inconclusive or single-target results for re-testing.

Archive

44 All colonies of appropriate morphology and with the *ttr* gene should be sub-cultured onto fresh mCASE agar

45 Incubate at 37°C for 18–24 h.

46 All pure growth should be harvested and placed into Microbank Cryopreservation Beads
- If culture is not pure, sub-culture until pure

47 All suspected *S*. Typhi (positive amplification for *ttr*, *tviB* and *staG*) should be archived separately from the Non-Typhoidal *Salmonella* isolates

48 Samples are stored at Ultra-Low Temperature (-80 °C), ensuring minimal freeze-thawing to ensure longevity.

Extraction for Whole Genome Sequencing

49 All isolates selected for whole genome sequencing will need to be retrieved from Cryobeads

50 Take one beads from the tube, and place onto a non-selective agar

51 Make a pool with the bead, and then streak the plates as normal for single colonies

52 Incubate at 37°C for 18–24 h.

53 Check for purity by taking one half of a colony for qPCR and subculture the other half (see above sections).

54 When selected isolates are pure, a 1 µL loop of bacterial growth from nutrient agar and inoculating 1.5 mL of nutrient broth, which was incubated at 37 ± 1°C for 18 to 20 hours

55 After incubation, samples were heat inactivated at 95 ± 2°C for 10 minutes

56 700 µL transferred into a deep 96-well plate

57 centrifuged for 20 minutes at 2,500 x g

58 The supernatant was discarded and replaced with 220 µL of ATL cell lysis buffer and 20 µL proteinase K

59 incubated at 60 ± 5 °C for 30 minutes

60 4 µL of RNase was added and incubated for 15 minutes at 37 ± 1°C

61 The plate was loaded onto the QiaSymphony

62 a DSP Virus/Pathogen mini-Kit was used with the default extraction profile on the machine

63 Yield and purity of each genomic DNA sample after extraction was determined using the Qubit 1x dsDNA Broad Range Assay kit

Protocol references

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