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ORIGINAL ARTICLE

Optimized methods for detecting *Salmonella* Typhi in the environment using validated field sampling, culture and confirmatory molecular approaches

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Abstract

Aims: This study evaluated detection methods for *Salmonella* Typhi (*S*. Typhi) in the environment, to establish a novel pathway from field sampling to isolation of viable organisms and molecular confirmation from complex environmental samples, thus enabling environmental surveillance of typhoid.

Methods and Results: Multiple media were assessed using clinical isolates from the Public Health England's (PHE) Culture collection. The culture pathway selected consisted of a primary 2% bile broth and secondary Selenite F broth, followed by modified Chromogenic Agar for Salmonella Esterase (mCASE). A qPCR assay was adapted from a validated *S*. Typhi PCR panel for confirmation of isolates, with comparison to biochemical and serological tests showing good specificity. Sampling locations in Blantyre, Malawi were used to compare sampling methods. Viable *S*. Typhi were isolated from a mixture of trap and grab river water samples on six occasions.

Conclusions: Culture of viable *S*. Typhi from environmental samples was possible using effective capture and culture techniques.

Significance and impact of study: Whilst several studies have attempted to detect *S*. Typhi from the environment, this is the first successful attempt to isolate the organism from river water since the 1980s. Supplementing clinical data with environmental screening offers the potential for enhanced surveillance, which might inform interventions and assess vaccination programmes.

KEYWORDS

bile broth, biofilm, biofilms, Identification, Malawi, mCASE, Moore swabs, PCR (polymerase chain reaction), river water, salmonella, selenite broth, typhoid, water

INTRODUCTION

Typhoid fever remains a public health problem of global concern, particularly in Low and Middle-Income Countries (LMICs) where water, sanitation and hygiene infrastructure are frequently inadequate (Feasey et al., 2015; Parry et al., 2002; Schwenk, 2020). Humans are the only known reservoir of *Salmonella enterica* serovar Typhi (*S*. Typhi). Whilst cases are ultimately transmitted from human to human, transmission may occur through direct or indirect exposure following excretion of the pathogen into the environment. This has been referred to as long-cycle transmission (Akullian et al., 2015; Baker et al., 2011; Gauld et al., 2018; González-Guzmán, 1989; Levine et al., 1982).

Whilst *S*. Typhi can often be readily detected in symptomatic patients by blood culture, environmental detection has proved more challenging. Gram Negative bacteria, including nontyphoidal *Salmonella* (NTS), coliform bacteria, *Escherichia coli* and other *Enterobacterales*, have been shown to suffer sublethal stress and injury when recovered from the environment which adds challenges to their isolation from samples (Rhodes & Kator, 1988); however, little is documented for *S*. Typhi due to its literature description as a human restrictive pathogen (Parry et al., 2002). It would, however, be advantageous to reliably detect *S*. Typhi from such samples, as environmental surveillance would considerably advance understanding of the epidemiology of typhoid fever and assist policy makers in establishing public health interventions (Sikorski & Levine, 2020). Previous attempts to identify how the environment is involved in this abiotic transmission have associated typhoid with water sources but they have not successfully cultured the organism (Baker et al., 2011). Without culture-based methods, the viability of *S*. Typhi and, therefore, its capability to cause human infection cannot be ascertained.

Whilst it has been possible to culture *S*. Typhi from environmental sources in the past, as has been described in high income settings in the mid-20th century (Moore, 1951; Moore et al., 1952), this has become restricted by microbiological laboratory capacity in endemic settings of the 21st Century (Sears et al., 1986) and a shift towards media production focusing on NTS due to its higher incidence in high-income countries (Kirchhelle et al., 2019; Majowicz et al., 2010; Oxford-Vaccine-Group, 2019; Stanaway et al., 2019). In recent years, molecular techniques have become the preferred method to detect environmental *S*. Typhi despite the culture-based identification approach remaining the gold standard (Mather et al., 2019). Molecular methods such as quantitative PCR (qPCR) have their own limitations and challenges, particularly in the endemic LMICs. There are concerns about sensitivity and specificity, and DNA from dead bacteria may persist and thus not be a true representation of viable bacteria that can cause infection (Zhou et al., 2018).

Despite the challenges of culture-based detection methods, there are clear advantages for their use. Where it has been successful (Sears et al., 1984), culture not only proves the presence of viable *S*. Typhi, but it also offers the opportunity to undertake further characterization of the organism, for example by whole genome sequencing (WGS). These techniques allow environmental isolates to be compared against the strains that cause human disease and thereby facilitate the investigation of transmission pathways and the associated epidemiology. In this study, we have optimized sample collection techniques, culture-based pathways and qPCR to establish methods that can reliably be used in endemic areas.

MATERIALS & METHODS

Methodologies were assessed and evaluated in the laboratory setting at the Public Health England (PHE) Food, Water and Environmental Microbiology Laboratory, London. They were then applied in the field in Blantyre, Malawi, where an outbreak of Typhoid fever, associated with use of river water for cooking and cleaning, began in 2011 (Gauld et al., 2020). Figure 1 and Table 1 summarize the workflow.

Strain information

Eighteen *S*. Typhi cultures from PHE's Gastrointestinal Bacteria Reference Unit (GBRU) were selected to represent strains recently identified from human cases of infection, isolated between 2009 and 2015 (Table 2). Isolates were from individuals who had recent travel history to Africa, the Indian subcontinent and South East Asia. Isolates included representatives of the H58 haplotype (genotype 4.3.1), which remains widespread in Malawi (Feasey et al., 2015) and included an isolate from a patient with recent travel history to Malawi.

The isolates were selected to challenge a range of media described in the scientific literature for the isolation of *Salmonella* species or *S*. Typhi (Tables 1 and 2). Isolates of different microbial species from PHE's culture collection were also utilized to determine media selectivity (Table 3).

Media Selection

Media were identified through a literature review and assessed on the availability, stability and safety status of

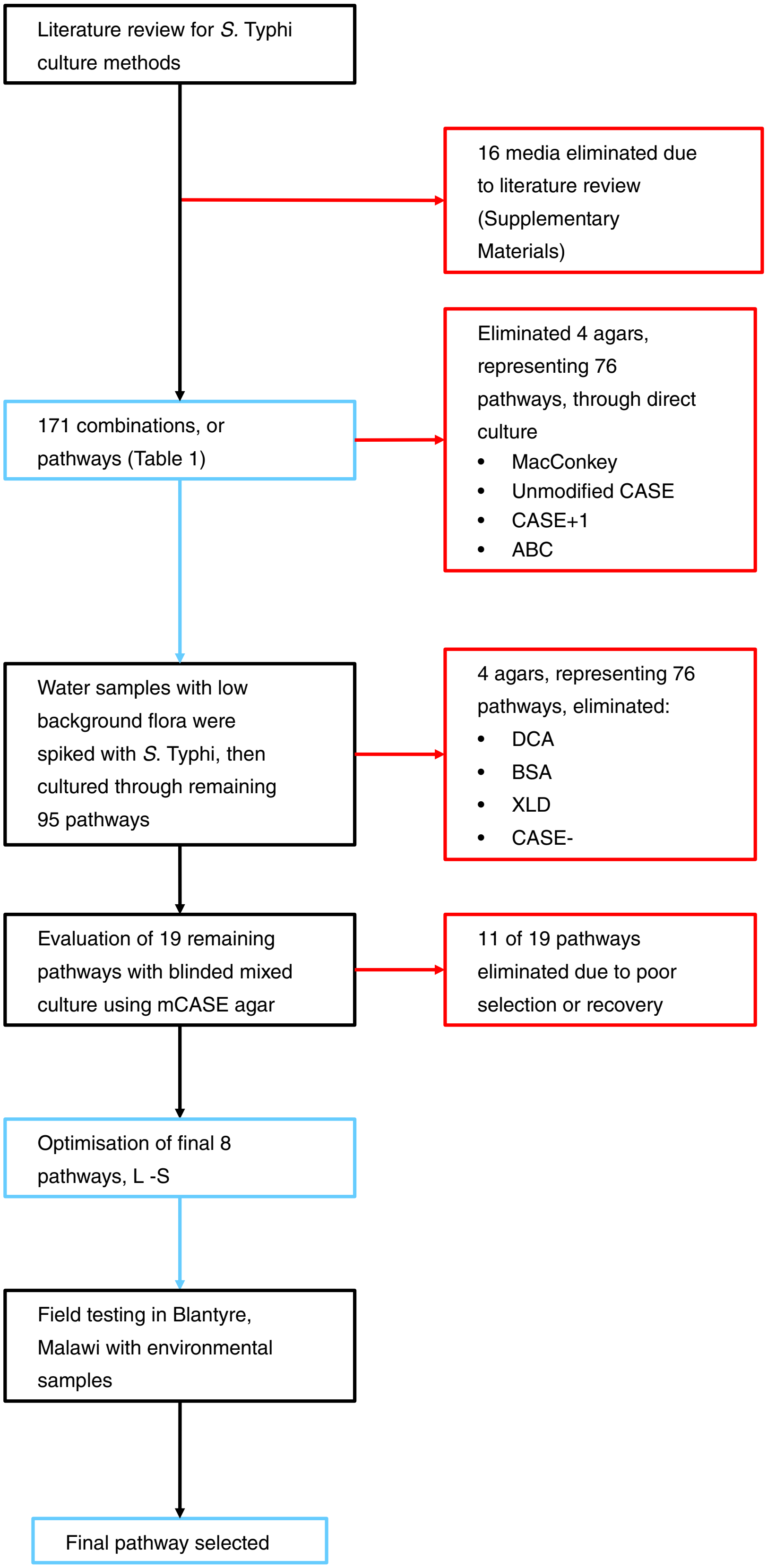

FIGURE 1 Workflow diagram showing the experiments and decisions taken to select the final pathway for *S.* Typhi culture. Black boxes, action taken; Red boxes, action outcome; Blue boxes, pathway information

their ingredients. A full list of the media eliminated without laboratory evaluation is provided in Table S1a,b.

Media performance was evaluated based on the growth of *S.* Typhi isolates (Table 2) on the following: xylose lysine deoxycholate agar (XLD; Oxoid); deoxycholate citrate agar (Hyne's media; Oxoid); bismuth sulphite agar (Wilson and Blair media; Oxoid); Harlequin ABC agar (Neogen); Chromogenic Agar for Salmonella

TABLE 1 The culture pathways evaluated in this study utilizing five broths and nine agars in 19 different broth combinations

Pathway	Primary broth	Secondary broth	Tertiary broth	Agar
A	BPW	SC	—	MacConkey
B	BPW	SC	Bile$^-$	CASE
C	SC +Bile$^-$	—	—	mCASE
D	SC	—	—	CASE+1
E	BPW	Bile$^-$	SC	CASE−
F	BPW	Bile$^-$	—	ABC
G	BPW	SC +Bile$^-$	—	DCA
H	Bile$^-$	BPW	SC	XLD
I	Bile$^-$	—	—	BSA
J	BPW +Bile$^-$	—	—	
K	BPW +Bile$^-$	Bile$^-$	—	
L	Bile$^-$	SC	—	
M	Bile$^+$	SC	—	
N	SC	Bile$^-$	—	
O	SC	Bile$^+$	—	
P	Bile$^-$	SF	—	
Q	Bile$^+$	SF	—	
R	SF	Bile$^-$	—	
S	SF	Bile$^+$	—	

Abbreviations: Bile$^-$, modified Enterobacteriaceae Enrichment broth; Bile$^+$, Bile broth with 0.2 g/L iron pyrophosphate; BSA, Bismuth Sulphite Agar; CASE−, CASE media with selective agents removed; CASE, Chromogenic agar Salmonella Esterase; CASE+1, CASE with one selective agent removed; DCA, Deoxycholate citrate agar; mCASE, CASE with the second selective agent removed; PW, Buffered Peptone Water; SC, Selenite Cystine; SF, Selenite F; XLD, Xylose lysine deoxycholate.

Esterase (CASE; Neogen); selenite cystine broth (SC; Sigma Aldrich and Oxoid); selenite F broth (SF; Neogen and Oxoid); buffered peptone water (Oxoid); bile$^-$ broth (modified Enterobacteriaceae Enrichment or EE broth; Neogen); bile$^+$ broth (bile$^-$ broth; Neogen, with 0.2 g/L iron pyrophosphate; Oxoid).

The base formulation of CASE was developed for the selective isolation and identification of *Salmonella* species. Identification is achieved using a dual chromogenic system utilizing esterase and β-glucosidase detection. Isolation is achieved by the incorporation of all the necessary target growth requirements (i.e., amino acids, vitamins and trace elements), as well as selective agents such as bile acids and metal and phosphate salts to inhibit common nontarget micro-organisms (Neogen, 2019). Additionally, two antibiotics are included in the base medium; the first was included to enhance selectivity against Gram positive and nontarget Gram negative *Enterobacterales*, whilst the second was to inhibit the growth of *Pseudomonas* spp. as these can exhibit strong esterase activity, giving false positives. The CASE media (Neogen) was modified for this project to generate three further agars to improve *S*. Typhi growth. The CASE− agar was the base agar with both antibiotics removed. The CASE+1 was the base agar with just the first antibiotic. The CASE+2 (later described as modified CASE; mCASE) was the base agar with just the second antibiotic.

Development of test pathways

Following a literature review, 171 test pathways were devised to represent possible combinations of the five broths and nine agars (Figure 1 and Table 1).

Qualitative studies

Candidate agars were initially screened with pure cultures of different strains of *Salmonella* serovariants, including *S*. Typhi and *E. coli* NCTC 9001; the *E. coli* was used as a negative control for those agars that selected or allowed identification of *Salmonella* spp. by inhibition or biochemical reactions. Each was prepared to a 0.5 McFarland standard (the inoculum) in Ringer's solution (Oxoid), which was then diluted to a 10^{-6} dilution. The dilutions were inoculated in triplicate onto each of the agars by spreading 100 μL onto each plate. After incubation at 37 ± 1°C for 18 ± 1 h, the growth was recorded as a qualitative score; +++ luxuriant growth, ++ good growth, + weak growth,

TABLE 2 *Salmonella* Typhi strains used in culture method evaluation experiments, which were provided from the Public Health England Gastro-intestinal Bacterial Reference Unit collection

Number	Isolated from	Year	Country of travel recorded	Antimicrobial susceptibility status	Haplotype (where available)	Sequence Type	eBurst Group	Accession ID[a]
1	Human faeces	2009	Nepal	A,C, Su, Tm, Nx, Cp	H58	1	13	SRR7165748
2	Human blood	2012	Malawi	A,C, Su, Tm	H58	1	13	SRR5949979
3	Human blood	2012	Vietnam	Nx, Cp		1	13	SRR1645294
4	Human blood	2012	The Democratic Republic of the Congo	A, Su,T, Tm		2	13	SRR1645361
5	Human blood	2013	Sudan			2	13	SRR5886991
6	Human faeces	2013	Niger	Nx, Cp		2	13	SRR5974884
7	Human faeces	2013	Nigeria			2	13	SRR7165353
8	Human blood	2014	Cameroon			1	13	SRR7165415
9	Human faeces	2014	India			2	13	SRR1967790
10	Human blood	2014	India	Nx, Cp		1	13	SRR1966683
11	Human blood	2014	Ethiopia			2	13	SRR3048982
12	Human blood	2014	Ghana	Tm, Nx, Cp		2	13	SRR7165399
13	—	2014	Zimbabwe	A,C,S, Tm, Nx, Cp		1	13	SRR1967049
14	Human blood	2015	Angola			1	13	SRR1963294
15	Human blood	2015	United Republic of Tanzania	A,C, Su, Tm		1	13	SRR1960208
16	Human blood	2015	Pakistan			1	13	SRR3048958
17	Human faeces	2015	India	A,C, Su, Tm, Nx, Cp		1	13	SRR1967675
18	Human blood	2015	Uganda	A,C, Su, Tm, Nx, Cp		1	13	SRR1967963

Abbreviations: A, Ampicillin; C, Chloramphenicol; Cp, Ciprofloxacin; Nx, Nalidixic Acid; Su, Sulphamethoxazole Tm, Trimethoprim.

[a]Accessible from https://www.ncbi.nlm.nih.gov/sra/ (last accessed 9th November 2020).

− absence of growth. The media that showed lower performance, no or weak growth, across the control strains were removed from further testing.

After removing the agar which allowed no or weak growth of *S.* Typhi, all culture pathways (Figure 1) of our proposed *S.* Typhi isolation protocol were used in conjunction with the remaining agar. Using spiked water samples that had low background flora from a local silt stream and tap water, 9 mL aliquots were inoculated with a 0.5 McFarland standard density of *S.* Typhi. Primary broths were inoculated with 1 mL of the water sample and incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into 9 mL of the secondary broth (Table 1), which was again incubated at 37 ± 1°C for 18 ± 1 h. After the incubation of the primary and secondary broths, a 50 μL volume was spread onto each agar (DCA, BSA, XLD, CASE− and mCASE) and incubated at 37 ± 1°C for 18 ± 1 h and growth was scored using the qualitative approach.

Incubation times were chosen based on manufacturer's recommendations and were not changed as a variable. This was due to the methods being developed for a surveillance programme that would not be able to operate at high sample numbers with incubation times requiring further processing same day.

Mixtures of known but undisclosed micro-organisms or mixed culture challenges

Mixtures of known but undisclosed culture collection micro-organisms were used to challenge the broth media pathways and mCASE (Tables 2 and 3; Table S3). Experiments were undertaken using a range of 18 NCTC strains and a wild-type *S.* Typhimurium, likely to challenge identification of suspected *S.* Typhi, these strains are listed in Table 3. These organisms were either other Salmonellae bacteria with similar morphology to *S.* Typhi (same colour on mCASE) or organisms likely to be found in the environment that might cause overgrowth on media. Combinations of these strains alongside a Malawian *S.* Typhi strain 2 (Table 3) were prepared as 10 blinded solutions by an independent laboratory worker, and these were used as inocula for each of the broth-based isolation pathways and agar to challenge selectivity.

TABLE 3 Reference strains used in the study and their growth characteristics on mCASE

Strain	NCTC[a]	WDCM[b]	Colour	Growth[c]
Bacillus cereus	7464	ATCC[d] 10876	Blue	+
Bacillus subtilis	10400	00003	Blue	+
Enterococcus faecalis	775	00009	Blue/Black	+
Escherichia coli	9001	00090/00155	Colourless	+++
Escherichia coli	13216	00202	White	+
Escherichia coli O157	12900	00014	Colourless	+++
Listeria innocua	11288	00017	Black	+
Listeria monocytogenes	11994	00019	Blue	+
Mycobacterium fortuitum	10394	ATCC 6841	Blue	+
Mycobacterium chelonae	946	ATCC[d] 35752	Blue	+
Pseudomonas aeruginosa	10662	00114	Blue	++
Raoultella planticola	9528	N/A	Black	+++
Saccharomyces cerevisiae	10716	00058	Blue	+
Salmonella Nottingham	7832	N/A	Blue/Green	+++
Salmonella Typhimurium	Wild-type	N/A	Blue/Green	+++
Staphylococcus aureus	6571	00035	Blue	+
Staphylococcus epidermidis	11047	00132	Blue	+
Vibrio furnissii	11218	00186	No Growth	−
Vibrio parahaemolyticus	10885	00185	Blue	+

Note: Key: +++ luxuriant growth, ++ good growth, + weak growth, − absence of growth.

Abbreviation: N/A, not applicable.

[a]National Collection of Type Cultures.

[b]World Data Centre for Micro-organisms.

[c]Based on at least triplicate data.

[d]American Type Culture Collection.

Enumeration studies

To assess the pathways that had previously performed best, enrichment was tested with the use of a known amount of inoculum to challenge the limit of detection (LOD). Using a Malawian strain, a suspension was made to a 0.5 McFarland standard density in Ringer's Lactate solution. A serial dilution was performed and the inoculum's CFU mL^{-1} was assessed through a spot-titre method (Miles et al., 1938) on Columbia blood agar (5% *v*/*v* horse blood; Oxoid) and incubating at 37 ± 1°C for 18 ± 1 h and quantified the following day. Using 1 mL of each of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of the inoculum were transferred into independent 9 mL preparations of each primary broth (Table 1). After completing each of the culture steps, these samples were again enumerated with the spot-titre method to confirm growth of the test strain.

After this initial assessment, the culture media's LOD was determined and used for further enrichment comparisons on the selected agar from previous assessments, mCASE. Subsequently, three biological replicates of the Malawian *S*. Typhi strain were prepared as independent inoculums per pathways L to S (Table 1), as described above. Each inoculum was enumerated as per the Miles, Misra and Irwin method, and 1 mL of the dilution identified as the LOD (10^{-6}) was transferred to a 9 mL volume of the primary broth for each pathway. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume of primary broth postincubation was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. An enumeration (Miles et al., 1938) was performed after preparation of the inoculum and after each broth (primary and secondary) incubation to allow quantification at each step.

Molecular confirmation of *S*. Typhi

Nucleic acid extraction was performed by boiling two colonies of pure growth in 500 μL of molecular grade water (Sigma) in a 1.5-mL centrifuge tube. After vortexing, the tube was heated at 95°C for 10 minutes in a dry block heater, after which, the tube was pulse centrifuged (five

TABLE 4 Primer and probe sequences used in multiplex quantitative PCR assays for the identification of *S*. Typhi

Gene	Gene purpose	Primer and probe sequences (5' – 3')		Accession number	Reference
ttr	Tetrathionate respiratory	Forward:	CTCACCAGGAGATTACAACATGG	AF282268	Hopkins et al., (2009)
		Reverse:	AGCTCAGACCAAAAGTGACCATC		
		Probe:	FAM-CACCGACGGCGAGACCGACTTT-BHQ1		
tviB	Vi polysaccharide biosynthesis protein	Forward:	TGTGGTAAAGGAACTCGGTAAA	NC_003198	Nair et al., (2019)
		Reverse:	GACTTCCGATACCGGGATAATG		
		Probe:	TET-TGGATGCCGAAGAGGTAAGACGAGA-BHQ2		
staG	Fimbrial protein	Forward:	CGCGAAGTCAGAGTCGACATAG	AL513382	Nga et al., (2010)
		Reverse:	AAGACCTCAACGCCGATCAC		
		Probe:	CY5-CATTTGTTCTGGAGCAGGCTGACGG-BHQ2		
sseJ	Secreted effector protein	Forward:	CGAGACTGCCGATGCATTTA	AF294582	Nair et al., (2019)
		Reverse:	GTACATAGCCGTGGTGAGTATAAG		
		Probe:	YY-TGGAGGCGGCCAGTAATATTGGTT-BHQ2		

seconds at 13,300 RPM/17,000g) and stored at 6 ± 2°C until real-time qPCR testing.

Molecular identification used a multiplexed adaptation of the Nair et al., (2019) qPCR focusing on the target genes *ttr*, *tviB*, *staG* and *sseJ* (Table 4). To utilize these assays in a multiplex format, new fluorophores were attached to the established probes enabling amplicon differentiation, and all 18 reference strains (Table 2) were screened against the assays (Supplementary Materials, Table S5).

The assays were performed in two stages: a duplex and a triplex format (Supplementary Materials, Table S4). The duplex screened isolates using *ttr*, the pan-*Salmonella* target and *sseJ*, found only in *S*. Paratyphi C and other nontyphoidal Salmonellae. All isolates that were *ttr* positive and *sseJ* negative would then be further screened by the triplex of *ttr*, *staG* (an established *S*. Typhi-specific target) and *tviB* (a target specific for *S*. Typhi and *S*. Paratyphi C) that was modified for use from gel electrophoresis assays to real-time PCR in Nair et al., (2019). However, the Vi antigen can also be found in some *Citrobacter freundii* (Snellings et al., 1981) with a 78% per identity match when a blast search is performed against *Citrobacter* spp., as such necessitating the use of *ttr* to confirm all isolates as a *Salmonella* spp.

A two-stage PCR was decided upon so that the sensitivity was not too adversely affected for the *S*. Typhi-specific primers and to minimize use of reagents as NTS are more common than *S*. Typhi, allowing samples that are *sseJ* positive, or *ttr* negative to be disregarded. Therefore, the *ttr*, *tviB* and *staG* would all need to generate an amplicon to determine the presence of *S*. Typhi DNA.

The assay used Takyon Low ROX Probe 2X dTTP blue MasterMix (Eurogentec); the primer and probe concentrations of each multiplex, DNA and total reaction volume are listed in Table S5. The fluorophores and quenchers are listed in Table 4.

Both the duplex and triplex assay formats were performed with 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 10 s using either the Applied Biosystems ViiA 7 and QuantStudio 7 platforms (Thermo Fisher Scientific), with 0.2-mL volume 96 well plates. Detection channels used were Blue (FAM), Green (TET), Yellow (Yakima Yellow) and Red (Cy5). Thresholds for the assay were set automatically as these gave reproducibly suitable values (between 0.08 ΔRn and 0.2 ΔRn) in the linear phase of exponential amplification. A positive was defined as amplification crossing the threshold between cycles 10 and 30.

Using 11 biological replicates of overnight culture of the Malawian strain of *S*. Typhi, suspensions were made to a 0.5 McFarland standard in Ringer's Lactate solution. DNA was extracted and serially diluted from 10^{-1} to 10^{-8}, and a standard curve was produced for *ttr*, *tviB* and *staG* as a triplex. These replicates were enumerated by culture prior to extraction to give a CFU mL^{-1} for each extract and serial dilution. With these results, the PCR efficiency (Eff%), Coefficient of Determination (R^2), the 50% and 95% limit of detections (LOD^{50} and LOD^{95}) and Limit of Quantification (LOQ) were calculated.

The Eff% was calculated by determining the slope of the average CFU mL^{-1} at each dilution as a Logarithmic 10 value and calculating the slope when plotted against the average CT value at each dilution and then using the formula (Svec et al., 2015):

$$\text{Eff\%} = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1.$$

The LOD was determined using the probit model (CLSI, 2012). The LOQ was determined using the coefficient of variance (Cv) between the replicates at each dilution, with the lowest dilution below a Cv of 25% selected (Kralik & Ricchi, 2017).

Phenotypic identification

Phenotypic identification was undertaken using API 20E (BioMerieux) and an anti-sera agglutination test, using the sera for O9 surface antigen, Vi antigen and Hd flagella antigen (Pro-Lab Diagnostics) on all isolates screened to confirm the results of the qPCR against traditional *Salmonella* typing methods. Anti-microbial susceptibility testing (AST) was also performed by disc diffusion method following EUCAST guidelines (EUCAST, 2021) on Mueller Hinton (Oxoid) agar to further differentiate isolates. The definition of multi-drug resistant (MDR) for *S*. Typhi is resistance to all three first line antimicrobials: co-trimoxazole (25 μg), chloramphenicol (30 μg) and ampicillin (10 μg) (Oxoid).

Field comparison of pathways P to S

The final candidate pathways L to S (Figure 1 and Table 1) were then deployed on field samples in Blantyre, Malawi to determine the most consistent culture method for isolation of *S*. Typhi from the environment.

Samples of 1 L water and soil were collected from and alongside four different water courses (rivers). The four sampling sites were selected using data provided from a case control study in which typhoid patient households were geospatially located (Gauld et al., 2019; Gauld et al., 2020). Sites were prioritised downstream of river junction points that had a large number of cases living upstream.

As per the Standing Committee of Analysts recommended method for *Salmonella* spp. (SCA, 2016), water samples were filtered through a 0.45 μM membrane under vacuum and the membrane was then placed into 10 mL of the primary broth. Soil was immersed in 18 mL of the primary broth in a 1:9 ratio, using two grams of soil. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. Using a 10-μL loop, the surface of an mCASE plate was inoculated to enable isolation of individual colonies. Two ten-fold dilutions were also made from the secondary broth after incubation, and 0.5 mL of the 10^{-1} and 10^{-2} preparations were spread over the surface of an mCASE plate using a sterile L-shaped spreader.

Confirmation was performed by qPCR and phenotypic methods, as described above.

Field studies in Malawi using final selected pathway

Field studies on the final selected pathway were established in **eight locations, and within each of these, there were 10 defined environmental sample collection sites. Four sampling sites were as described above, with additional sites including a sewage plant, which eluted into the Mudi river and three communities: Zingwangwe, Ndirande and Mbayani (map in Supplementary Materials, Figure S4). At each of the 10 sample collection sites per location, up to five different sample types were collected based on availability. Targeted sample types included 1L water collections, food (vegetables, fruits and spices), soil, riverbed rock samples coated in biofilms (2–5 cm in diameter) and Moore swabs (Moore, 1951; Sikorski & Levine, 2020).

Pathway P was selected for the field studies. Water samples were filtered through a 0.45 μM membrane and then placed into 10 mL of the primary broth. All solid samples were cultured at a ratio of 1:9 of solid sample to media, as such two grams of soil was immersed in 18 mL and rock biofilms were immersed in 20 mL of the primary broth (biofilm was scraped off in culture media, but stone not removed); similarly, five grams food and Moore swabs were immersed in 50 mL. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. Using a 10-μL loop, the surface of an mCASE plate was inoculated to enable isolation of individual colonies. Three ten-fold dilutions were also made from the secondary broth after incubation, and 0.5 mL of the 10^{-2} and 10^{-3} preparations were spread over the surface of an mCASE plate using a sterile L-shaped spreader.

Confirmation of presumptive *S*. Typhi was by qPCR. If qPCR positive, phenotypic analysis was undertaken using an API 20E biochemical panel, by serology and AST. Sampling methods were split into grab (1L water samples) and trap samples (Moore swabs and biofilms) and then compared using a Fisher's exact test to compare efficacy between sampling approaches.

RESULTS

Thirty media were selected from the literature review and of these, 16 were eliminated as being unsuitable (Supplementary Materials, Table S1a,b). The reasons for elimination included logistics, shelf-life and a reliance in traditional media on the absence of lactose fermentation or the production of hydrogen sulphide (H_2S) to distinguish colonies despite these being unreliable for the identification of *Salmonella* spp. (Aksoysan et al., 1981; Kunz & Ewing, 1965; Wilson, 1948).

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Preliminary screening of agars to reduce candidate pathways

Direct culture of *S*. Typhi strains on the nine candidate agars led to four agars being eliminated based on quality of growth and selectivity: MacConkey; Unmodified CASE; CASE+1; ABC (Figure 1). MacConkey agar was eliminated as it had broad selectivity for enteric, Gram-negative bacteria making it difficult to distinguish *Salmonella* spp. from other *Enterobacterales*. Unmodified CASE and CASE+1 gave poorer growth than the other two formulations: mCASE and CASE− (Figure 2). The other Neogen chromogenic agar, ABC, also proved to be less favourable as the α-galactosidase chromogen gave false negative results for some *S*. Typhi strains.

The 95 remaining pathways were challenged through spiked water experiments (Figure 1). It was not possible to sufficiently distinguish *S*. Typhi from the natural background flora using four of the agars, DCA, BSA, XLD and CASE−, so these were eliminated, removing 76 pathways; descriptions of colony morphology for each of the agar can be found in Supplementary materials (Table S2). The mCASE agar, in contrast, gave consistent recovery of *S*. Typhi through the remaining 19 pathways.

Mixed culture challenges

Using five blinded mixes of culture collection strains (Table 3) and *S*. Typhi, all remaining 19 pathways (Table 1) were evaluated using only the mCASE agar. This led to the removal of a further 11 pathways, A to K, due to their use of nonselective broth media and subsequent overgrowth of *S*. Typhi by the other genera represented in the culture collection strains. It was also observed that colony morphology was impacted as a result of the order in which broth media was used.

For the remaining eight pathways, L to S, a further blinded study was performed in which two out of five mixed cultures contained *S*. Typhi. For the two *S*. Typhi mixes, all eight pathways had isolates with typical morphology on mCASE (Figure 3). In pathway M and Q, the *S*. Typhi could not be isolated as pure culture and only identified as a *Salmonella* species through phenotypic identification.

Quantification of broth recovery, pathways L to S

Primary broths used for pathways L to S were seeded using a Malawian clinical strain of *S*. Typhi. Growth was quantified from the primary broth and the secondary broth after incubation. It was identified that culture pathways had to be seeded with a minimum of 100 CFU mL^{-1}, the 10^{-6} dilution, for consistent growth to be observed. Therefore, we determined the limit of detection of our culture pathway to be 10^2 CFU mL^{-1} as inocula of 10^1 CFU mL^{-1} did not allow for sufficient growth within the primary or secondary broth incubations to allow robust detection.

Twenty-four biological replicates were used to perform all eight pathways in triplicate, to determine their reproducibility and selective advantage. As presented in Figure 4 (additional information in Supplementary Materials Figure S1 and Table S4), pathways L, P and S demonstrated consistent growth from inoculum through both primary and secondary broth culture, where all replicates showed similar performance and the secondary broth showed good growth after the primary broth. Pathways M, Q, N, R and O all demonstrated a reduction in the level of growth after the transition from primary to secondary broth. Analysis of the difference of log10 for pathway L, P and S (Figure 5) showed similar growth between the inoculum to primary and primary to secondary stages of the experiment. After a review of the growth experiment

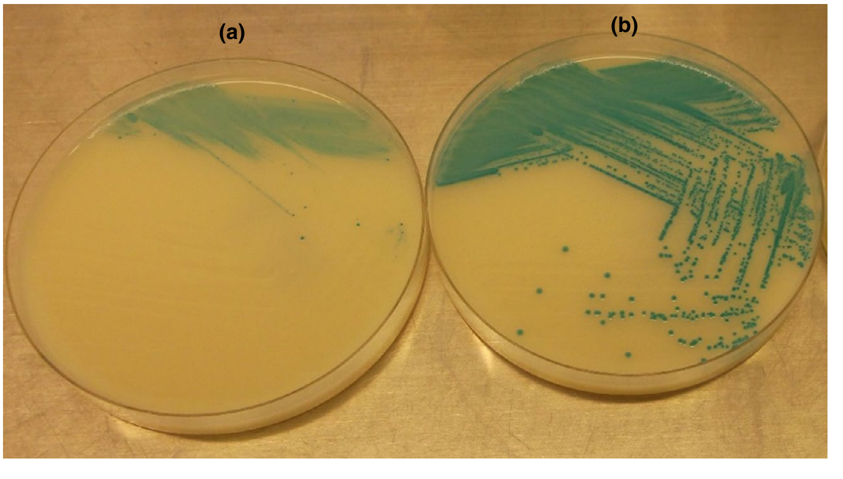

FIGURE 2 Comparison of *S*. Typhi growth luxuriance on (a) unmodified CASE and (b) modified CASE (mCASE)

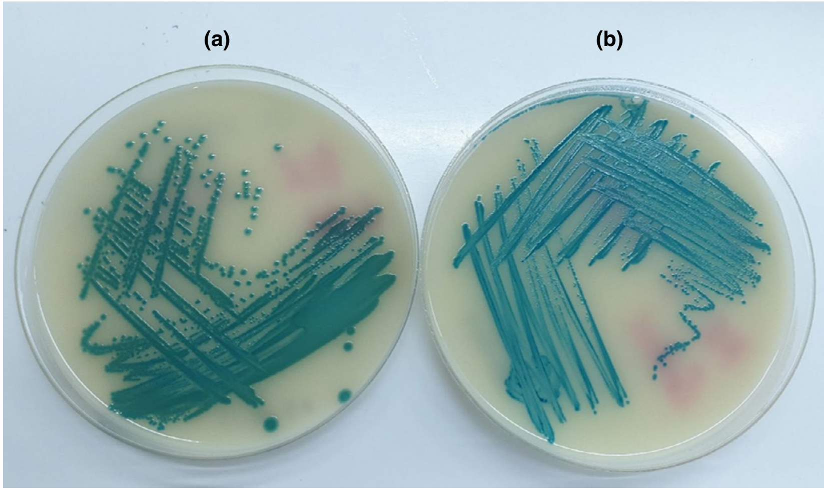

FIGURE 3 Growth of (a) *Salmonella* Typhimurium and (b) *S.* Typhi on mCASE to demonstrate the difference in colony colour and morphology. The *S.* Typhi colonies metabolise the chromogen, generating a bluer colony colour and a smaller colony size than other tested serovariants, with translucent halo. This difference is subtle but appreciable with experience

results alongside consideration of the availability of the broth used, pathway L was eliminated as selenite cystine broth was not consistently available from suppliers, with some discontinuing the product. The pathways P and S were taken through into field trials in Malawi to confirm that performance was equivalent in naturally contaminated samples.

PCR validation

A modification of the real-time qPCR assays as described by Nair et al., (2019) was developed in this study to allow isolate identification and quantification of *S.* Typhi from environmental samples. All 18 of the *S.* Typhi strains (Table 2) used to challenge this assay amplified with *ttr*, *staG* and *tviB*, whilst the *S.* Nottingham (Table 3) and a *S.* Typhimurium strain amplified *ttr* and *sseJ*. *Escherichia coli* strain NCTC 9001 did not cross-react with any primer target.

Using the Malawian strain of *S.* Typhi (Table 2), the primer efficiency (Eff%), coefficient of determination (R^2), limit of detection (LOD) and limit of quantification (LOQ) were calculated (Supplementary Materials, Figure S2). The Eff% and R^2 values fell within 100% and 110%, and 0.99 to 1, respectively: *ttr* 106.6%, 0.993; *tviB* 101.2%, 0.997; *staG* 108.7%, 0.995. The LOD was determined using the probit model analysis method and determined that the LOD^{50} was 4.84×10^1 CFU mL^{-1}, 6.85E $\times 10^2$ CFU mL^{-1} and 1.18×10^2 CFU mL^{-1} for primer pairs for *ttr*, *tviB* and *staG*, respectively. The LOD^{95} was 3.60×10^2 CFU mL^{-1}, 3.61×10^3 CFU mL^{-1} and 8.97×10^2 CFU mL^{-1}, respectively (Supplementary Materials, Figure S2). The LOQ for all assays was 1.74×10^3 CFU mL^{-1}.

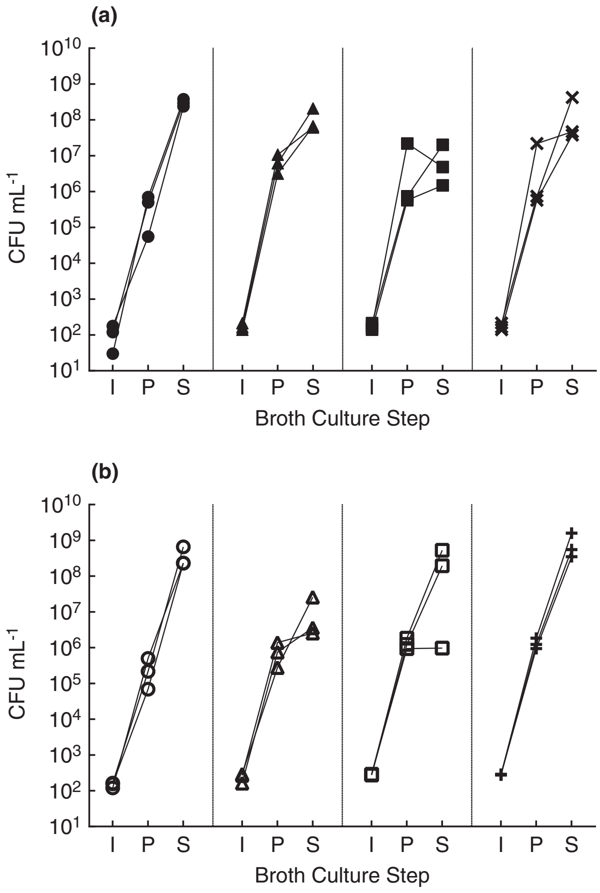

FIGURE 4 *Salmonella* Typhi growth across pathways L to S, representing colony counts at inoculation (I), postincubation of the primary (P) and secondary (S) enrichment broths. Data is divided between the selenite cystine (a) and selenite F (b). A. shows pathways L, M, N and O (Left to right): bile$^-$ to selenite cystine (●), bile$^+$ to selenite cystine (▲), selenite cystine to bile$^-$ (■) and selenite cystine to bile$^+$ (x). B. shows pathways P, Q, R and S (left to right): bile$^-$ to selenite F (❍), bile$^+$ to selenite F (Δ), selenite F to bile$^-$ (□) and selenite F to bile$^+$ (+)

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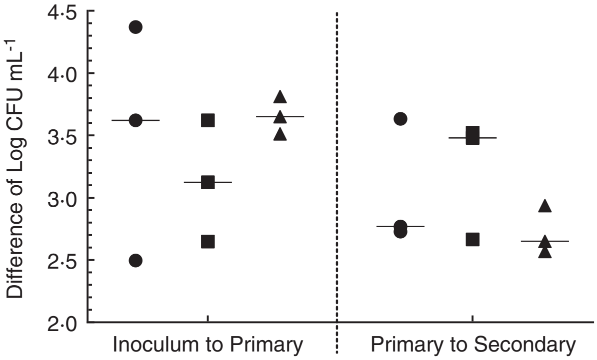

FIGURE 5 Graph showing the difference of log for inoculum to postincubation primary (I to P) broth colony counts and postincubation primary to secondary (P to S) broth colony for pathways L (●), P (■) and S (▲)

In situ use of culture pathways in Malawi

Over a three-month period of sample collection (March to May 2019), the pathways P and S (Table 3) were used in parallel in the field. No *S*. Typhi was isolated from the 27 water samples collected. Observations from growth on the mCASE identified that pathway P demonstrated better recovery of NTS than pathway S. Pathway S also allowed greater growth of contaminating organisms including, *E. coli*, swarming bacteria and fungi, which appeared to impact NTS recovery and therefore was likely to reduce the success of isolating *S*. Typhi.

Field application of Pathway P

Between June 2019 and January 2020, 592 samples were collected across the eight sampling locations in Blantyre, Malawi. These sample types could be separated into two categories: grab and trap samples. We define grab samples as comprising of 1 L water samples (532 collected of a total 592 samples), which provide a snapshot of the *S*. Typhi status of the river collected in the bottle at the particular time and location the sample is taken; and trap samples (60/592), as objects that remain in the river for a longer period (48–72 h), concentrating material *in situ* and increasing the likelihood for the target organism to be captured. Trap samples included Moore swabs (19 swabs out of 60 trap samples), which capture particulates and organisms within the gauze over the period of deployment due to river flow; and rocks covered in biofilms (41/60) (Table 5), which also capture organism due to the nature of biofilms. The number of *S*. Typhi positives from grab samples (1/532) were then compared to the number of positives from the trap samples (5/60) using Fisher's Exact test, and a p value of 5.07×10^{-5} was calculated (Table 5). This demonstrates statistically that trap samples are more likely to be positive for *S*. Typhi (risk ratio from Table 5 = 44.33, i.e., trap samples were 44.33 times as likely to be positive for S. Typhi in this study's field experiment). No *Salmonella* spp. was isolated from food or soil.

TABLE 5 Number of each sample type collected between June 2019 and January 2020, and the number that were positive by culture for *S*. Typhi. It provides the number of grab samples (1L waters sampling) and trap samples (Moore swabs and biofilms) collected

Sample type	Number negative	Number positive
Water	531	1
Total grab samples	*531*	*1*
Moore swab	16	3
Biofilm	39	2
Total trap samples	*55*	*5*

Note: Comparing the positivity of grab to trap samples using Fisher's Exact test a p value of 5.07×10^{-5} was calculated with a risk ratio of 44.33, meaning trap samples are 44.33 times more likely to be positive.

In this study, geospatial data were combined with current local knowledge of river usage and access points to identify field sampling sites. Of the six positive samples, *S*. Typhi was isolated from: one sample collected from a hotspot located by the geospatial data; four samples from areas with geolocated cases but not identified as a high priority area; and one collected from a site selected independently from the model. Of these samples, five were collected from a river with a busy market located upstream, which demonstrates the importance of combining modelled and observation data (Baker et al., 2011; Gauld et al., 2019; Mirembe et al., 2019; Pitzer et al., 2019).

Additionally, a further 121 unique isolates of NTS were identified with the qPCR assay by testing positive for *ttr* and *sseJ*, but negative for *staG* and *tviB*. Of these, 55 were from the 1L grab samples, 31 from Moore swabs, 5 from biofilms and a further 30 from other sources (algae, soil and other water surface plants and debris).

Confirmation of environmental isolates

The qPCR was performed on all presumptive *Salmonella* spp. isolates, of which six had *ttr*, *tviB* and *staG* genes detected but not *sseJ*, identifying them as *S*. Typhi. The six *S*. Typhi isolates were then screened by API 20E and antisera agglutination for additional confirmation. For all six, the API 20E returned one of two profiles, 4005540 and 4405540, both of which indicate a 99.9% identification for *S*. Typhi. All six isolates were associated with an agglutination reaction against all three of the O9, Vi and Hd antigen target sera. In addition, their AST profiles were determined, and resistance identified to ampicillin, chloramphenicol and sulfamethoxazole.

DISCUSSION

This study describes a comprehensive approach to environmental detection of *S.* Typhi. Our work has addressed the whole process from the suitability of sample type through to isolate confirmation, considering field sampling, sample processing, bacterial enrichment and isolation. This study is important because it provides a method to evidence long cycle typhoid transmission, which is not as well quantified as short-cycle transmission, but which plays a key role in the epidemiology of typhoid fever (Gauld et al., 2018).

We reviewed 30 culture media for the isolation of *S.* Typhi (Supplementary Materials, Table S1a), 12 were disregarded due to their shelf-life and reliability of supply chain, as well as their reliance on unreliable distinction methods (absence of lactose fermentation and/or H_2S production), compared to more robust modern media, such as chromogenic agars. Of the remaining 19, selenite-based media provided best selectivity. Selenite F broth was our preferred option, due to a lack of availability of selenite cystine, with little difference in performance between the two being evident. The toxicity of selenite-based media is a disadvantage, both in its powder form for inhalation and the danger it poses to aquatic life and environments. This can be controlled through comprehensive risk assessment and limitation of the volume to 10 mL per sample with appropriate disposal systems in place (Neogen, 2019). Further, mCASE was modified specifically for this project to achieve a more optimal performance for *S.* Typhi; with commercially produced media focussing on the isolation of NTS from food, water and environmental samples, additional work could be pursued in the future to modify each of the media used to increase their performance for *S.* Typhi recovery. Additionally, further variations could be assessed, such as incubation times with the use of items like incubator shakers.

Due to the harshness of selenite media and the low concentrations of *S.* Typhi in contaminated water samples, a pre-enrichment broth that provided selectivity for *Salmonella* spp. was considered a requirement. As part of our evaluation of culture pathways, 2% bile broth was selected. The bile broth, a modified version of EE broth with the Typhi-inhibitory malachite green removed, was selected for the recovery of sublethally injured *S.* Typhi. This pre-enrichment broth also mimicked the known exposure to bile in the human host during *S.* Typhi infection of the duodenum (Parry et al., 2002). When compared with more traditional enrichment media for sublethal injury, such as buffered peptone water, 2% bile broth gave much better recovery whilst also providing some selective pressure due to the bile salts within the medium, showing its utility for *S.* Typhi isolation. Further, the addition of iron to this broth to increase *S.* Typhi cell recovery had the unintended consequence of increasing the growth rate of competitive organisms, and assisting in their survival when subcultured into selenite media, making isolation on agar more challenging.

The purpose of utilizing real-time qPCR in this study was to provide a low-cost, high-throughput confirmation tool for isolates of *Salmonella* spp., including *S.* Typhi. The assay used four primer pairs previously described (Nair et al., 2019). It was deemed essential that a multi-target approach be taken to comprehensively determine the identification of an isolate as *S.* Typhi as it has been hard to establish a single primer pair with requisite sensitivity and specificity (Nair et al 2019). We decided upon *ttr* gene primers as a pan-salmonellae assay that confirms genus of the isolate. The *staG* primers have been used exclusively in some studies for direct detection of *S.* Typhi from environmental samples; however, the *sta* operon, in which *staG* is located, is known to be detected in other salmonellae such as *S.* Sendai, Gallinarum (Pu3 and Pu4), Dublin, Enteritidis and Derby (Townsend et al., 2001). Whilst *tviB* is more specific, only being found in *S.* Typhi and Paratyphi C, there have been reports of S. Typhi pathogenic strains without the *SPI-7* pathogenicity island, within which the gene for the Vi antigen is encoded (Baker et al., 2005). Lastly, as we were performing PCR on single picks, *sseJ*, which is not found in *S.* Typhi was used to detect non-typhoidal serovariants. All isolates that were *staG* positive, but *tviB* negative were *sseJ* positive and phenotypically confirmed to be NTS. In contrast, all isolates that were *ttr*, *staG* and *tviB* positive and *sseJ* negative were phenotypically confirmed to be *S.* Typhi. Our data therefore support this primer combination. The qPCR's efficiency and reproducibility fell within the acceptable range and are therefore suitable to be used as a confirmation tool for *S.* Typhi identification. Further, the LOD and LOQ were in the range of a well-performing qPCR assay (Taylor et al., 2019).

Whilst other studies have isolated *S.* Typhi from sewage and heavily contaminated domestic use water (Roy et al., 2016), here we present the first description for the isolation of *S.* Typhi from river water and river-borne environmental samples since the 1980s (Sears et al., 1986; Sears et al., 1984). We previously developed a methodology that places the genomes of clinically isolated organisms in the spatial context of human cases of infection to predict environmental hotspots of typhoid transmission (Gauld, 2020; Gauld et al., 2019). Geolocating the homes of typhoid fever patients allowed for the development of an optimized method for field sampling, targeting the environmental sampling in areas of known transmission and large numbers of cases. These hotspots provided locations where new and traditional sampling methodologies could be applied to determine whether a capture (grab) method or capture and concentration (trap) method was most effective for *Salmonella*. Whilst we detected *S*. Typhi using both trap and grab samples, trap samples were significantly more effective. Due to intermittent shedding of the organism, Moore swabs were created to "trap" the organism when sampling and used to great effect in previous outbreaks, including for organisms other than *S*. Typhi (Sikorski & Levine, 2020). This is despite variation in the volumes in culture media used, reducing volumes of selenite F media from typical volumes of 200–500 mL down to 10 mL (Sears et al., 1986; Sikorski & Levine, 2020).

Potential loss of Moore swabs presents a problem with only three-quarters of the swabs deployed in this study being recovered; further, two visits to the field are required per Moore swab (deployment and collection 48–72 h later). To reduce sample loss, we attempted and successfully demonstrated the use of environmental biofilms as a sampling tool for *S*. Typhi. Naturally occurring and available in any river water source, this could prove to be a suitable, low-cost, widely available environmental sample. Rocks covered in biofilms, or biofilm scrapings cannot be lost in the same way as a Moore swab, or other deployed tool for long-term collection. The only disadvantage is we do not know the duration that the *S*. Typhi has resided in the biofilm; however, as we are not aware that typhoid has a true reservoir outside of humans, we consider this a minor limitation when compared to cost savings.

In application of the model for this study, there was an assumption used that the 2015–2016 clinical case data would be reflective of current disease presentation across the city, which was potentially not reliable. As no comparator testing process was included in the study, the sensitivity and specificity of this approach cannot be determined due to the lack of a reference standard. However, the analytical validation of each stage of the processing indicates this combination is likely a sensitive methodology for the detection of *S*. Typhi in environmental samples. Additionally, linking in more recent spatially referenced case data would assist in further testing of these methods, in the case that spatial patterns of incidence have changed over time.

This study describes a holistic approach to *S*. Typhi capture, concentration, culture and confirmation in the environment. We combine historical experience of environmental surveillance of *S*. Typhi with molecular approaches, to improve our chances of isolating it from complex environmental matrices. With the increase in antimicrobial resistant strains of *S*. Typhi, the identification of long-cycle reservoirs for typhoid are important to allow for targeted intervention programmes to reduce incidence, and thereby, burden of the disease. We believe this approach will support impact assessment following typhoid conjugate vaccine introduction. The culture-based approach also allows for the identification of NTS, which may be of interest due to the high prevalence of invasive nontyphoidal salmonella disease in regions with endemic *S*. Typhi. The use of an environmental surveillance programme would not only allow identification of areas where interventions could be implemented but could also be used as an effective tool for the monitoring of vaccination programmes world-wide, and their impact on the local transmission and exposure of typhoid.

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CONFLICT OF INTEREST

No conflict of interest declared by any author.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article, further data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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