




Real-Time PCR Assay for Differentiation of Typhoidal and Nontyphoidal *Salmonella*

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ABSTRACT Rapid and accurate differentiation of *Salmonella* spp. causing enteric fever from nontyphoidal *Salmonella* is essential for clinical management of cases, laboratory risk management, and implementation of public health measures. Current methods used for confirmation of identification, including biochemistry and serotyping as well as whole-genome sequencing analyses, take several days. Here we report the development and evaluation of a real-time PCR assay that can be performed directly on crude DNA extracts from bacterial colonies for the rapid identification of typhoidal and nontyphoidal *Salmonella*.

KEYWORDS Nontyphoidal salmonellae, real-time PCR, *Salmonella*, whole-genome sequencing, typhoidal salmonellae

Our novel 2-h assay identifies the genus *Salmonella* by detecting the *ttr* gene, encoding tetrathionate reductase, and defines typhoidal *Salmonella* by the detection of *Salmonella enterica* serovar Typhi and Paratyphi-specific gene combinations. PCR assay performance was determined using 211 clinical cultures of *Salmonella* (114 nontyphoidal and 97 typhoidal strains) and 7 clinical non-*Salmonella* cultures. In addition, the specificity of the assay was evaluated *in silico* using a diverse in-house collection of 1,882 *Salmonella* whole-genome sequences. The real-time PCR results for 218 isolates and the genomic analysis of the 1,882 isolates produced 100% sensitivity and 100% specificity (based on a 7-gene profile) for identifying typhoidal *Salmonella* compared to the *Salmonella* whole-genome sequencing identification methods currently used at Public Health England (PHE).

This paper describes a robust real-time PCR assay for the rapid, accurate identification of typhoidal and nontyphoidal *Salmonella* which will be invaluable for the urgent screening of isolates from symptomatic individuals, for the safe processing of isolates in laboratories, and for assisting the management of public health risks.

Salmonella is a diverse genus of gastrointestinal pathogens that cause a wide spectrum of diseases from self-limiting gastroenteritis (nontyphoidal salmonellae [NTS]) to systemic enteric fever (typhoidal salmonellae [TS]: *Salmonella enterica* serovar Typhi and serovars Paratyphi A, B, and C). Salmonellosis is global, but typhoidal salmonellae are found mainly in sub-Saharan Africa and South Asia, where enteric fever is endemic (1), although detailed local surveillance data from regions of endemicity remain poor (2). A current concern is the increase in bacteremia (and focal infections) associated with multidrug-resistant NTS infection in sub-Saharan Africa. In high-income countries such as the United Kingdom, invasive NTS infection is mainly confined to immunocompromised hosts, so the major risks are local outbreaks of NTS through poor food hygiene and typhoidal infections associated with travel to regions of endemicity.

Diagnostic hospital microbiology laboratories make only a presumptive identification of *Salmonella* spp.: they do not usually hold a sufficient range of specific antisera

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for full identification and rapid identification systems, such as matrix-assisted laser desorption/ionization–time of flight mass spectroscopy, are unable to fully identify *Salmonella*. In reference laboratories where definitive microbiological methods for the identification of *Salmonella* by serology and biochemistry (3) are available, the turn-around times are often lengthy because of weak expression of the somatic (O), flagellar (H), and Vi polysaccharide surface antigens leading to incomplete or incorrect identification of the serovars. Whole-genome sequencing (WGS) for *Salmonella* (4) has simplified the process for identifying *Salmonella* serovars substantially but still takes days rather than hours. Currently there are no rapid diagnostic tests for informing clinical and public health management of enteric fever or for ensuring that *Salmonella* isolates are processed appropriately with respect to laboratory safety.

In the United Kingdom, salmonellosis is a significant public health problem causing morbidity and financial loss due to sickness and absenteeism until clearance from infection for certain professions. The clinical management of salmonellosis patients depends on diagnosis. Enteric fever is treated with antibiotics, but nontyphoidal *Salmonella* gastroenteritis is usually self-limiting. Invasive disease needs to be treated with antibiotics specific to the strain causing infection. In addition, the processing of isolates or specimens in the laboratory from patients with suspected diarrheal infection depends on the identification of the causal agent. In the United Kingdom, microorganisms that pose a risk to human health are classified into one of four hazard groups based on the ability to infect healthy humans. The classification of these organisms allows the risk they pose to laboratory and health care workers to be controlled by implementing safety measures at the appropriate containment level (5). *S. Typhi* and *S. Paratyphi* A, B, and C are classified as hazard group 3 (HG3) pathogens in the United Kingdom and many regions globally (but not in all countries, such as those in North America), requiring processing in a specialized containment level 3 (CL3) laboratory (5). It is clear, therefore, that in order to treat patients effectively and protect health care and laboratory staff, the rapid identification of a patient as being infected with a typhoidal salmonella is critical.

At present there is no single rapid method to identify all TS, even though genomic data on the presence or absence of genes in both typhoidal and nontyphoidal *Salmonella* are in abundance. The *ttr* gene, encoding tetrathionate reductase, has been used as a PCR gene target to detect and identify *Salmonella* since it is present in all *Salmonella* spp. (6). However, it is not intended to distinguish typhoid and nontyphoidal subspecies. A few potential candidate genes for identifying TS have been described previously. For example, the *tvjB* gene, encoding a Vi polysaccharide capsule, which is present in *S. Typhi* and *S. Paratyphi* C (7) but not in *S. Paratyphi* A or *S. Paratyphi* B, can identify a subset of TS but does not distinguish *S. Typhi* or *S. Paratyphi* C (8). In order to differentiate *Salmonella* serovars causing enteric fever, additional genes are required. Nga et al. proposed using *SPA2308*, encoding a hypothetical protein, for the detection of *S. Paratyphi* A and STY0201 (also known as the *staG* gene), encoding a putative fimbrial protein, for the detection of *S. Typhi* in clinical blood samples via PCR (9). Connor et al. suggested that *S. Paratyphi* B (TS) could be distinguished from *S. Java* (NTS) using two genes encoding type III secretion system (TTSS) effector proteins, *sseJ* and *srfJ* (10), with *S. Paratyphi* B possessing only *srfJ* but *S. Java* possessing both *sseJ* and *srfJ*. However, as *sseJ* is also absent in *S. Typhi* and *S. Paratyphi* A, this gene cannot be used to differentiate all TS serovars or used alone as an NTS marker. A potential gene target for *S. Paratyphi* C identification is the *SPC0869* target, a gene encoding a hypothetical protein, shown to be present only in *S. Paratyphi* C (8). The use of this gene requires further assessment to ensure that it is a unique target among the *S. Paratyphi* C population, as only five serovars were investigated in the study by Liu et al. (8).

The design of a PCR assay to identify *Salmonella* and differentiate NTS and TS requires a multitargeted approach with defined gene profiles and rigorous validation. The aim of this study was to develop and validate a real-time PCR assay to distinguish TS (HG3) and NTS (HG2) and identify specific serovars of TS using traditional molecular and bioinformatic techniques.

TABLE 1 Number and type of *Salmonella* serovars tested via molecular PCR

Sequence type(s)	eBURST group(s)	Serovar or species	Serotype	Hazard group ^b	No. (n = 218)
Typhoidal <i>Salmonella</i>					
1, 2, 2173	13	<i>Salmonella</i> Typhi	9,12[Vi]:d:-	3	61
85, 129	11	<i>Salmonella</i> Paratyphi A	1,2,12:a:[1,5]	3	15
86	5	<i>Salmonella</i> Paratyphi B	1,4,[5],12:b:1,2	3	15
146	20	<i>Salmonella</i> Paratyphi C	6,7,[Vi]:c:1,5	3	6
Total					97
Nontyphoidal <i>Salmonella</i> isolates					
11, 183	11, 183	<i>Salmonella</i> Enteritidis	1,9,12:g,m:-	2	14
19, 34, 36	19, 34, 36	<i>Salmonella</i> Typhimurium	1,4,[5],12:i:1,2	2	14
10	10	<i>Salmonella</i> Dublin	1,9,12[Vi]:g,p:-	2	14
43, 88, 2545	43, 88, 0	<i>Salmonella</i> Java	1,4,[5],12:b:1,2	2	13
2902, 3226, 139, 145	0, 0, 6,6	<i>Salmonella</i> Choleraesuis	6,7;c:1,5	2	6
Variable ^a	Variable ^a	Selection of <i>Salmonella</i> spp. from GeneFinder analysis ^a	Variable; see Table S1	2	53
Total					114
Non-<i>Salmonella</i> isolates					
245, 152, 252, 7375	CC245, 152, 145, 0	<i>Shigella flexneri</i> , <i>S. sonnei</i> , <i>S. boydii</i> , <i>S. dysenteriae</i>	3a, NA, O6, O1	2	4
11, 29, 40	CC11, 21, 40	EPEC (<i>eae</i>), STEC (<i>stx1a</i> or <i>eae stx2a</i>) ^c	O55:H12, O77:H1, O157:H7	2	3
Total					7

^aA random selection of hazard group 2 ST containing sporadic gene targets was chosen.

^bAs defined in the Advisory Committee on Dangerous Pathogens (ACDP) guidelines (5).

^cEPEC, enteropathogenic *E. coli*; STEC, Shiga toxin-producing *E. coli*.

MATERIALS AND METHODS

Bacterial strains. A total of 211 *Salmonella enterica* subspecies I isolates, received at the Gastrointestinal Bacterial Reference Unit (GBRU), Public Health England (PHE) between 2008 and 2017 (Table 1), were used in this PCR study. Representative NTS isolates from the two most common serovars, *S. Enteritidis* and *S. Typhimurium*, as well as serovars that can be difficult to distinguish from TS isolates by traditional methods, including *S. Dublin*, *S. Java*, and *S. Choleraesuis*, were selected (Table 1). Assay specificity was further investigated by the inclusion of four *Shigella* isolates (*Shigella flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*) and three *Escherichia coli* isolates (containing either *eae* or *stx* genes) as representatives to test the specificity against other *Enterobacteriaceae* that are occasionally misidentified by referring clinical laboratories using automated identification platforms (see Table S1 in the supplemental material).

***Salmonella* whole-genome sequence data.** A total of 1,882 *Salmonella* whole-genome sequences (including the 211 *Salmonella* isolates), representing the diversity of salmonellae tested by the GBRU, were included in an *in silico* validation of the specificity of the selected target genes (Fig. 1). This data set included representative sequence types (ST) of the 19,221 strains validated and reported at the GBRU between 2016 and 2017. The strains selected included all subspecies of *Salmonella* and the common (3 or more isolates received between 2016 and 2017 at PHE) *Salmonella* serovars, representing in total 477 different sequence types (Table 2 and Table S1).

DNA extraction and real-time PCR assays. DNA from 218 isolates was extracted via a crude extraction method in which a single colony from MacConkey agar (Thermo Fisher Scientific, Waltham, MA) was inoculated into 490 μ l of sterile distilled water in a screw-cap microtube (Eppendorf, Hamburg, Germany) and placed in a boiling water bath for 20 min. Primers and probes for *ttr* (detection of all *Salmonella*), *tviB* (detection of *S. Typhi* and *S. Paratyphi* C), *SPA2308* (detection of *S. Paratyphi* A), and *staG* (detection of *S. Typhi*) were based on previously published studies (Table 3). Primers and probes for *SPC0869* (detection of *S. Paratyphi* C) and *sseJ* and *srfI* (detection of *S. Paratyphi* B) were designed using the PrimerQuest Tool V8 (<https://www.idtdna.com/PrimerQuest/Home/Index>) using sequences obtained from the NCBI nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>) (Table 3).

The real-time PCR reported here was carried out as seven individual multiplex reactions but also worked as duplex and triplex PCR assays with interchangeable primer and probe targets (and probe dyes) depending on the target gene combination required (data not shown, but master mix details are in Data Set S1). The master mix for the multiplex assay consisted of 12.5 μ l of Takyon Low Rox probe master mix (Eurogentec, Liège, Belgium), 8 μ l of nuclease-free water, 0.5 μ l each of 20 μ M forward and reverse primers, 1 μ l of 5 μ M probe, and 2.5 μ l of DNA in a final reaction volume of 25 μ l. A negative control was run with each PCR using 2.5 μ l of nuclease-free water for the template (Severn Biotech, Kidderminster, UK), and the following positive controls were used: NCTC 8385 for *S. Typhi* (*ttr*, *tviB*, and *staG*), NCTC 11803 for *S. Paratyphi* A (*ttr* and *SPA2308*), NCTC 8299 for *S. Paratyphi* B (*ttr* and *srfI*), NCTC 96 for *S. Paratyphi* C (*ttr*, *sseJ*, *tviB*, and *SPC0869*), NCTC 6676 for *S. Enteritidis* (*ttr* and *sseJ*), and NCTC 14013 for *S. Typhimurium* (*ttr*, *sseJ*, and *srfI*). The PCR was run on an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The conditions for the PCR were an initial activation of 95°C for 3 min,

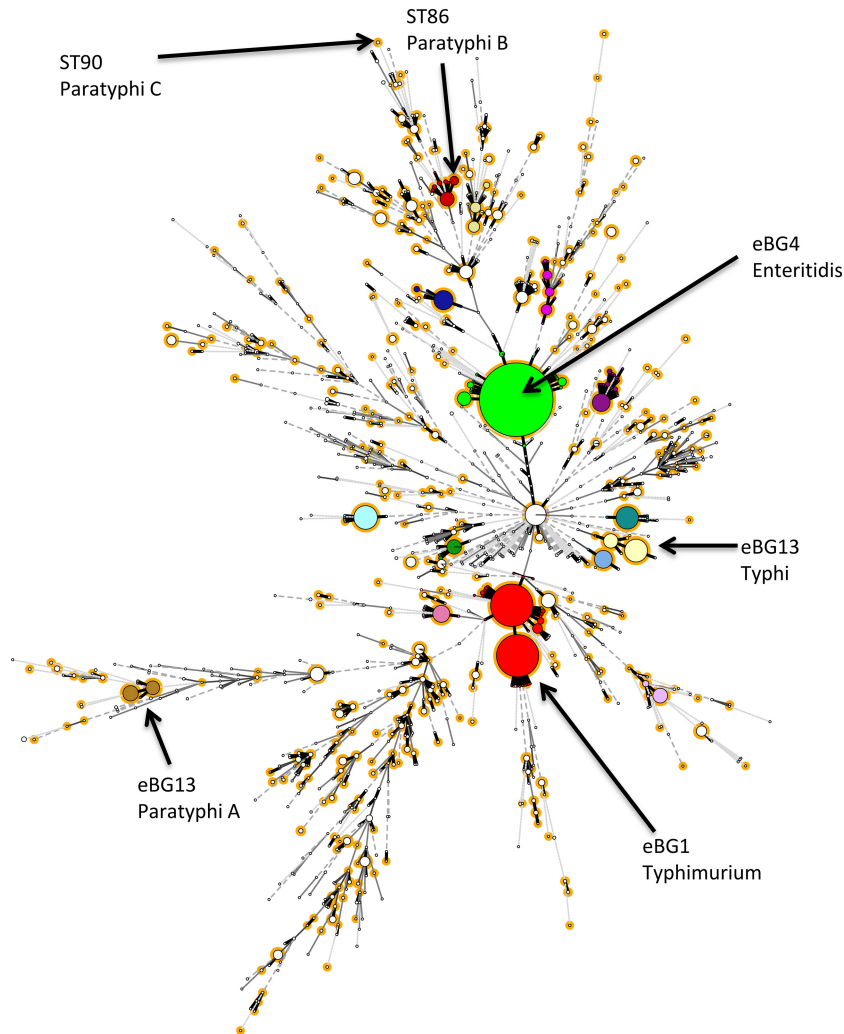


FIG 1 Selection of representative strains to test *in silico*. Shown is a population structure of *Salmonella* received at PHE between 2016 and 2017 and strains tested by PCR in this study, totaling 19,221 strains. Strains are color coded by main eBURST groups (eBG). Representative strains (highlighted in orange) from each sequence type within an eBG containing 3 or more isolates were selected for *in silico* detection of the seven genes (*ttr*, *sseJ*, *srfJ*, *tviB*, *SPC0869*, *SPA2308*, and *staG*).

followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. A positive result was assigned when a threshold cycle (C_T) value was achieved between 12 and 30 with a threshold set at a maximal fluorescence (ΔR) of 0.03.

Identification of TS and differentiation from NTS were based on a profile of seven genes (Table 4). The molecular and/or *in silico* PCR identification was compared with the original identification of the serovar obtained via a combination of WGS identification, phenotype, and serology carried out by the *Salmonella* laboratory as described previously (4) (Table S1).

PCR assay evaluation. The sensitivity and specificity of the *ttr*, *sseJ*, *srfJ*, *tviB*, *staG*, *SPA2308*, and *SPC0869* primers and probes (Table 4) used in the real-time PCR assays were calculated according to the method of Martin (11).

In addition, PCR assay specificity was assessed by *in silico* genomic analysis using a diverse in-house WGS data set covering the population structure of *Salmonella* (Fig. 1). A total of 1,882 *Salmonella* sequences (Fig. 1 and Table S1), including the 211 *Salmonella* isolates tested by PCR, were screened for the presence of seven target genes (*ttr*, *sseJ*, *srfJ*, *tviB*, *staG*, *SPA2308*, and *SPC0869*) using a PHE in-house bioinformatics tool called GeneFinder (developed by M. Doumith [unpublished data]). This tool takes paired-end Illumina FASTQ reads and aligns them to a reference sequence of the target genes, as a multi-FASTA file (accession numbers in Table 3), using Bowtie2 v2.1.0 (12) and Samtools v1.0.18 (13) and determines metrics such as coverage, presence of indels (insertions or deletions), amino acid alterations, the presence of single nucleotide polymorphisms, and overall similarity of the test sequence to the reference gene sequence. Target genes were designated present when sequences achieved a detection threshold of 80% sequence similarity to the reference gene, apart from *ttr*, for which the threshold was set at 70% sequence similarity, due to the size and variability of this particular gene (in-house validation).

TABLE 2 Number and type of *Salmonella* serovars tested via *in silico* (GeneFinder) analysis

Type of organism	No.
<i>Salmonella</i> Typhi	556
<i>Salmonella</i> Paratyphi A	315
<i>Salmonella</i> Paratyphi B	53
<i>Salmonella</i> Paratyphi C	6
HG2 serovars	952
Nonsalmonellae	7
Total	1,889
Sequence types	480
Subspecies I	1,821/1,889
Subspecies II	14/1,889
Subspecies IIIa	14/1,889
Subspecies IIIb	29/1,889
Subspecies IV	3/1,889
Subspecies V	1/1,889

Any discrepant results between GeneFinder and the PCR were investigated further by assembling the sequence data using Spades v3.1.1 to default parameters and examining the variability of primer and probe binding sites.

Assay reproducibility was determined by testing 20 of the 211 *Salmonella* isolates in triplicate. Precision was evaluated by the standard deviation of C_T values of 10 replicates of each of the positive controls for each target. Each target was assessed individually and as a multiplex in separate assay runs by different individuals and had the threshold set at 25% of the maximal fluorescence (ΔR) of each respective target.

RESULTS

Comparison of real-time PCR and current PHE methods for distinguishing NTS and TS. Of the 211 *Salmonella* isolates subjected to PCR identification, all gave the expected gene profile identification (Table 5 and Table S1), matching the original identification, except for 3 *S.* Typhi isolates in which the *tviB* gene was not detected.

TABLE 3 Primer and probe sequences used for each gene target with the fluorescent dye colored^a

Gene	Name	Sequence (5'–3')	GenBank accession no.	Reference
<i>ttr</i>	<i>ttr</i> _F	CTCACCAGGAGATTACAACATGG	AF282268	6
	<i>ttr</i> _R	AGCTCAGACCAAAAAGTGACCATC		
	<i>ttr</i> _P	FAM-CACCGACGGCGAGACCGACTTT-BHQ1		
<i>sseJ</i>	<i>sseJ</i> _F	CGAGACTGCCGATGCATTTA	AF294582	This study
	<i>sseJ</i> _R	GTACATAGCCGTGGTGAAGTATAAG		
	<i>sseJ</i> _P	CY3-TGGAGGCGGCCAGTAATATTGGTT-BHQ1		
<i>srfJ</i>	<i>srfJ</i> _F	CTGTCTGTATAGCGTGGAAAGAG	AF231759	This study
	<i>srfJ</i> _R	GTCCACCAGGCCATCTTTAT		
	<i>srfJ</i> _P	JOE-CGGCAGGGTATGGATGAGATGGAG-BHQ1		
<i>tviB</i>	<i>tviB</i> _F	TGTGGTAAAGGAACTCGGTAAA	NC_003198	7 (modified)
	<i>tviB</i> _R	GACTTCCGATACCGGATAATG		
	<i>tviB</i> _P	JOE-TGGATGCCGAAGGTAAGACGAGA-BHQ1		
SPC0869	SPC0869_F	CTGGCTGACACATGAACAAATC	NC_012125	This study
	SPC0869_R	CCTGAGAACGAGTCAGGTTTAC		
	SPC0869_P	CY5-TGTACGACTGCAACGCCAAAGTC-BHQ2		
SPA2308	SPA2308_F	ACGATGATGACTGATTTATCGAAC	FM200053	9
	SPA2308_R	TGAAAAGATATCTCTCAGAGCTGG		
	SPA2308_P	CY5-CCCATAACAATTCATTCTTATTGAGAATGCGC-BHQ2		
<i>staG</i>	<i>staG</i> _F	CGCGAAGTCAGAGTCGACATAG	AL513382	9
	<i>staG</i> _R	AAGACCTCAACGCCGATCAC		
	<i>staG</i> _P	FAM-CATTTGTTCTGGAGCAGGCTGACGG-BHQ1		

^aThe color of the reporter is related to spectrum of detection; quenchers are in bold. FAM, 6-carboxyfluorescein; BHQ, black hole quencher; F, forward primer; R, reverse primer; P, probe.

TABLE 4 Gene profiles for the identification of *S. Typhi* and *S. Paratyphi* from other serovars^a

Profile	<i>Salmonella</i> identification	<i>ttr</i>	<i>sseJ</i>	<i>tviB</i> ^b	<i>srfJ</i>	<i>SPC0869</i>	<i>SPA2308</i>	<i>staG</i>
1	<i>S. Typhi</i>	+	–	+/–	–	–	–	+
2	<i>S. Paratyphi</i> A	+	–	–	–	–	+	–
3	<i>S. Paratyphi</i> B	+	–	–	+	–	–	–
4	<i>S. Paratyphi</i> C	+	+	+	–	+	–	–
5	NTS serovar ^c	+	+	–	+/–	–	–	–
6	Non- <i>Salmonella</i> spp.	–	–	–	–	–	–	–

^a+, present; –, absent.

^b*tviB*⁺ means that the strain is genotypically Vi positive.

^cA proportion of NTS serovars will be positive for the *ttr* gene and a combination of targets that do not match any of the TS profiles (Table S1).

This was confirmed by *in silico* analysis (see below). Previously described TS gene targets *SPA0869*, *staG*, and *SPA2308* were found sporadically (41/114 [35%] of the NTS tested; 2 isolates had two TS gene targets present), confirming that use of single targets to differentiate TS from NTS is not appropriate (Table 6 and Table S1). None of the 7 target genes were detected in the four shigellae or three *E. coli* isolates that were tested.

Whole-genome sequencing *in silico* analysis. For the 1,882 *Salmonella* isolates subjected to *in silico* analysis, identification based on gene profiles (Table 4) matched the original identification but did highlight that individual gene targets could be found sporadically across the *Salmonella* population. *In silico* analysis identified 952/1,882 nontyphoidal *Salmonella* isolates that were positive for *ttr* and a combination of other TS gene targets (Table 6 and Table S1), designated profile 5 (Table 5). None of the gene profiles of these isolates matched the designated TS profiles (profiles 1 to 4), and thus, our interpretation is that *ttr*-positive strains with a profile not matching the TS profiles should be classified as NTS (Tables 4 and 5 and Table S1).

As with the real-time PCR assay, the *Salmonella* processed via *in silico* analysis identified the three SPI-7-negative *S. Typhi* isolates. The real-time PCR and GeneFinder correctly identified the deletion of this gene.

In this study, 8 of the 1,882 sequences were positive by PCR and yet negative for the same gene by GeneFinder. Further *in silico* analysis revealed that the genes concerned had an intact primer and probe binding site, thus confirming the PCR result, but variation outside these regions resulted in average similarity values below the GeneFinder threshold value (Table S1).

Reproducibility and precision of PCR assay. Reproducibility was assessed by performing the PCR 3 times on 20 isolates. The results indicated that the PCR was reproducible for differentiating between NTS and TS and for the identification of serovars within TS (Table S1). The precision analysis demonstrated that five out of the seven gene targets were considered precise (i.e., standard deviation < 0.167). The following average *C_T*s (with standard deviations in parentheses) were obtained for the indicated genes: *ttr*, 25.12 (0.154); *sseJ*, 23.59 (0.127); *srfJ*, 24.51 (0.179); *tviB*, 25.01 (0.115); *staG*, 24.97 (0.121); *SPC0869*, 25.68 (0.142); and *SPA2308*, 20.59 (0.248). Both *srfJ* and *SPA2308* have standard deviations above the value of 0.167, which is considered

TABLE 5 Summary of gene profile results

Profile	<i>Salmonella</i> serovar (no. tested)	Expected genes present	Result	Matches (%)
1	<i>Salmonella Typhi</i> (556)	<i>ttr</i> , (<i>tviB</i> ^{+/-}), <i>staG</i>	556	100
2	<i>Salmonella Paratyphi</i> A (315)	<i>ttr</i> , <i>SPA2308</i>	315	100
3	<i>Salmonella Paratyphi</i> B (53)	<i>ttr</i> , <i>srfJ</i>	53	100
4	<i>Salmonella Paratyphi</i> C (6)	<i>ttr</i> , <i>sseJ</i> , <i>tviB</i> , <i>SPC0869</i>	6	100
5	NTS serovar (952)	<i>ttr</i> (plus combination of any of the following not fitting the above profiles: <i>sseJ</i> , <i>srfJ</i> , <i>SPC0869</i> , <i>SPA2308</i> , and <i>staG</i>)	952	100
6	Non- <i>Salmonella</i> spp. (7)	Negative for all genes	7	100

TABLE 6 Summary of individual gene target results

<i>Salmonella</i> strain (no. tested)	No. with indicated gene						
	<i>ttr</i>	<i>sseJ</i>	<i>tviB</i>	<i>srfJ</i>	<i>SPC0869</i>	<i>SPA2308</i>	<i>staG</i>
<i>Salmonella</i> Typhi (556)	556	0	553	0	0	0	556
Atypical <i>Salmonella</i> Typhi (3)	3	0	0	0	0	0	3
<i>Salmonella</i> Paratyphi A (315)	315	0	0	0	0	315	0
<i>Salmonella</i> Paratyphi B (53)	53	0	0	53	0	0	0
<i>Salmonella</i> Paratyphi C (6)	6	6	6	0	6	0	0
NTS serovar ^a (952)	952	938	0	380	19	50	41
Non- <i>Salmonella</i> spp. (7)	0	0	0	0	0	0	0

^aThe combination of genes present was heterogeneous; please see Table S1 for details.

not precise. The explanation for this is that these two primer/probe sets are more susceptible to variation due to the *srfJ* reverse primer having no G/C's in the GC clamp, therefore increasing the possibility of variable binding to the target gene. The *SPA2308* forward primer has less than 40% GC content, making it more thermally variable, and both reverse primer and probes do self-anneal and form hairpins. This is the case as the *SPA2308* gene has a very low GC content of 32.25% and as a result will lead to more variable results. Another important note is that this validation process occurred using boiled cells as the DNA extraction method (as this is the intended use for rapidity) and there is always the possibility of slight levels of PCR inhibition, in comparison to using purified DNA, which will also affect the precision results. The lower precision level did not affect the molecular PCR in practice and was deemed suitable for use.

Sensitivity and specificity. Sensitivity and specificity were based on the 7 gene profiles (and not individual gene markers) detected by real-time PCR and GeneFinder (Table 5). It showed 100% sensitivity and specificity for the detection of TS compared to the routine reference identification by WGS and serotyping.

DISCUSSION

This study describes for the first time a robust real-time PCR assay for the specific identification of each of the four TS serovars, *S. Typhi* and *S. Paratyphi* A, B, and C; this assay is 100% reliable (Fig. 1, Tables 5 and 6, and Table S1). This assay was validated as a monoplex PCR providing the flexibility to use individual targets of interest and is now in use routinely at PHE. The rapid (2-h) turnaround time of this PCR assay has potential for expediting reporting of results to customers (urgent TS confirmatory requests from hospitals, coupled with antimicrobial susceptibility testing) and the management of suspected cases of typhoid fever. Many laboratories are moving away from serology (due to the cost of maintaining sera and results being subjective, especially for the Vi polysaccharide sera used to identify *S. Typhi*) and are depending on whole-genome sequencing identification (sequence type), which can take up to 7 to 10 days. Public Health England stopped routine serology in 2015 (4).

With additional optimization, the application of this assay could be extended to direct testing of clinical specimens (blood and stool) as well as food, water, and environmental specimens. This would further increase the value of the assay, although such use may risk the possibility of fewer isolates being referred to reference laboratories for further characterization, leading to loss of typing for surveillance purposes (including antimicrobial resistance monitoring) as well as outbreak detection and investigation. Thus, it is essential that isolates continue to be isolated and referred to reference laboratories.

Recently, there has been an emphasis on using rapid and accurate molecular tools (e.g., real-time PCR) for the detection of TS in low- and middle-income countries where the disease is endemic for epidemiological surveillance purposes. Data from these surveillance studies will help countries make evidence-based decisions to facilitate control and prevention (vaccine) measures and allows rapid outbreak detection (14–18).

Many assays for identifying TS have been described previously, but these usually involved single gene targets with much lower specificity and sensitivity, as the assays

were not tested against a large set of diverse *Salmonella* serovars or were aimed at just one or two of the TS serovars and some of the assays were carried out by conventional PCR (9, 19–21). However, these important studies have provided input for the selection of candidate gene targets in designing a gene profile-based PCR assay; the validation of this PCR assay was strengthened by the use of WGS sequence data for high-throughput testing on a more diverse collection of *Salmonella* isolates.

In silico analysis has its limitations if relied on as the sole method. Although PHE utilizes a multilocus sequence type (MLST)-based approach with genomic data for *Salmonella* identification (4), other organizations may use a gene-based approach for *Salmonella* identification, and the use of set thresholds with *in silico* testing in the current study on target genes (i.e., at what threshold is the test positive) may need to be flexible depending on the gene. Unlike detection via PCR, the entire target gene is evaluated using *in silico* analysis, and therefore, we can draw conclusions on the presence or absence of the target gene. However, selecting a threshold value (and therefore a percent identity of a match) for which a gene is considered present or absent can be difficult. Discrepancies between real-time PCR and genomic detection of target genes occur when a gene has less than the set threshold of sequence similarity. There were initially eight negative gene results using GeneFinder that were positive by PCR. These were due to a lower percentage of gene similarity and below the 80% set threshold (Table S1) and were positive for the presence of the gene (matching the PCR result). When mismatches between PCR and *in silico* methods occur, explicit consideration is required to ascertain if the PCR primer/probe binding region is intact and how much of the gene is present. Specifically, in our targets, *ttr* showed a large range of variability among isolates in terms of sequence similarity to the reference gene, with five of eight of these samples having <80% *ttr* sequence similarity. After assessing the primer/probe binding sites of the genes, there were no discrepancies between GeneFinder and the PCR assay.

This current study showed that 17 of the 952 NTS isolates were positive for only a single gene target (*ttr* gene) (Table S1) and belonged to *Salmonella* subspecies III, IV, and V. Therefore, most NTS contain one or more of the other genes markers normally associated with TS (Table 6). This highlights that a single gene target method is not appropriate for distinguishing between typhoidal and nontyphoidal *Salmonella*, with a gene profile-based method being more accurate for identification and differentiation of TS. The reassuring finding, however, is that not one of the 935 NTS had the same gene profile as the TS (Tables 4 and 5 and Table S1).

Another notable observation is that three *S. Typhi* isolates from Pakistan lacked the 134-kb SPI-7 pathogenicity island harboring the *viaB* operon (*tvi* genes, associated with the production of the Vi capsule). Although rare, the absence of the SPI-7 pathogenicity island, including the *tvi* region, in *S. Typhi* has previously been described (7). This is potentially an important public health finding, as the current typhoid Vi polysaccharide vaccine stimulates immunity against the Vi capsule. It is known that SPI-7-negative (Vi-negative) *S. Typhi* can cause typhoid fever (22), so there is a need to monitor the loss of the SPI-7 island in regions of endemicity where *S. Typhi* vaccination programs are being conducted (22). The assay described here could be used to monitor the emergence of Vi-negative *S. Typhi* through the emergence of *ttr*- and *staG*-positive *tviB*-negative strains.

Conclusion. In conclusion, ours is the first real-time PCR assay that can rapidly distinguish between typhoidal, i.e., *S. Typhi*, Paratyphi A, Paratyphi B, and Paratyphi C, and nontyphoidal *Salmonella* serovars. The assay has the ability to be implemented in diagnostic and reference laboratories globally as a safe and cost-effective way of differentiating salmonellae as well as to be used for epidemiological surveillance purposes.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00167-19>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

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