SUPPLEMENT ARTICLE

Detection of Typhoidal and Paratyphoidal *Salmonella* in Blood by Real-time Polymerase Chain Reaction

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Background. The gold standard for diagnosis of enteric fever caused by *Salmonella* Typhi or *Salmonella* Paratyphi A or B is bone marrow culture. However, because bone marrow aspiration is highly invasive, many hospitals and large health centers perform blood culture instead. As blood culture has several limitations, there is a need for novel typhoid diagnostics with improved sensitivity and more rapid time to detection.

Methods. We developed a *clyA*-based real-time polymerase chain reaction (qPCR) method to detect *Salmonella* Typhi and *Salmonella* Paratyphi A simultaneously in blood. The sensitivity and specificity of this probeset was first evaluated in vitro in the laboratory and then in a typhoid-endemic population, in Karachi, Pakistan, and in healthy US volunteers.

Results. We optimized a DNA extraction and real-time PCR-based method that could reliably detect 1 colony-forming unit/mL of *Salmonella* Typhi. The probe set was able to detect clinical *Salmonella* Typhi and *Salmonella* Paratyphi A strains and also diarrheagenic *Escherichia coli*, but not invasive *E. coli* or other invasive bacteria. In the field, the *clyA* qPCR diagnostic was 40% as sensitive as blood culture. However, when qPCR-positive specimens were considered to be true positives, blood culture only exhibited 28.57% sensitivity. Specificity was ≥90% for all comparisons and in the healthy US volunteers. qPCR was significantly faster than blood culture in terms of detection of typhoid and paratyphoid.

Conclusions. Based on lessons learned, we recommend that future field trials of this and other novel diagnostics that detect typhoidal and nontyphoidal *Salmonella* employ multiple methodologies to define a "positive" sample.

Keywords. diagnostic; blood; *Salmonella*; typhoid; PCR.

Collectively, invasive infections by *Salmonella* species are estimated to cause >30 million new illnesses annually [1–3]. The majority of enteric fever cases (caused by *Salmonella enterica* serovars Typhi and Paratyphi A) occur in South and Southeast Asia, whereas invasive infections with nontyphoidal *Salmonella* (iNTS) are primarily observed in sub-Saharan Africa [4–6]. Clinical diagnosis of invasive *Salmonella* infection is complicated due to similarity of symptoms to other febrile illnesses such as malaria, dengue, and rickettsioses. Ambulatory healthcare facilities in endemic settings frequently lack laboratory-based diagnostics, resulting in the majority of diagnoses being made clinically and antimicrobials given empirically [4, 6–8].

Early and reliable detection is an essential step in delivering successful patient care, controlling disease spread, and determining treatment outcome. Additionally, the ability to reliably implicate *Salmonella* as a cause of disease would permit improved estimates of burden at a national and global level, thereby creating a case for future investment in treatment and preventive

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Clinical Infectious Diseases® 2015;61(S4):S241–50

DOI: 10.1093/cid/civ726

methods and ultimately enabling accurate evaluation of such interventions [9]. Diagnostic tests that can detect invasive *Salmonella* require different attributes depending on the goal. A diagnostic for patient care should be rapid, sensitive, and specific; economical; simple to operate; and ideally able to detect antibiotic resistance. A diagnostic that would be used to measure disease burden should have the following attributes: sensitive and specific; economical; and able to differentiate between *Salmonella* Typhi, *Salmonella* Paratyphi A (in Asia), *Salmonella* Typhimurium, *Salmonella* Enteritidis (in Africa) and other *Salmonella* serovars.

The most widely available methods of laboratory diagnosis of *Salmonella* bloodstream infection rely on direct culture [10]. However, culture-based methods require that viable bacteria be present in detectible quantities at the time of specimen sampling; this has proven to be a major hurdle in diagnosis of *Salmonella* as reports of bacterial burden at systemic sites during invasive *Salmonella* infection are universally low and many patients have received antibiotics prior to their arrival at the hospital [11, 12].

Bacterial burden varies throughout the course of infection but is reported to be similar for both typhoidal and nontyphoidal *Salmonella*, which establish an intracellular niche at a density of <1 colony-forming unit (CFU)/mL of peripheral blood or 10 CFU/mL of bone marrow [13–15]. Bacterial burden is an order of magnitude higher in the bone marrow than in peripheral blood and is increased relative to that of peripheral blood during antibiotic therapy and relapse of infection [13–15]. Blood culture exhibits approximately 60%–80% sensitivity during the first 7 days of infection. The sensitivity drops to 20%–30% at subsequent time-points and although positively correlated with blood draw volume, the recommended sample volume of 10 mL can be difficult to obtain from acutely ill children [10, 16, 17].

Culture of bone marrow aspirates is considered the most accurate method of identifying acute *Salmonella* Typhi infection [18], but requires technical skill and appropriate equipment, is invasive, and does not eliminate the need for laboratory culture and subsequent serological identification. Bone marrow culture has not been fully evaluated as a diagnostic for invasive NTS infections. For both typhoidal and nontyphoidal *Salmonella*, most diagnostic laboratories use blood culture methodology for detection. Laboratory methods most commonly used for blood culture employ an automated blood culture instrument followed by traditional bacteriology identification and susceptibility testing [19].

Diagnostic targets for new typhoid detection tests include bacterial proteins, metabolites, and nucleic acid using technologies such as mass spectroscopy, polymerase chain reaction (PCR), and DNA hybridization [20–23]. Of particular note, Zhou and Pollard [24] have shown that preincubation of blood in bacteriological medium can improve sensitivity of molecular techniques. Several groups have taken advantage of this and are currently evaluating novel typhoid molecular diagnostic assays following preincubation. Other methods used to diagnose acute *Salmonella* Typhi infection involve detection of host response markers. Some of the most popular tests are the Widal, Tubex-TF, Typhidot IgG, and Typhidot IgM tests, which have low to moderate sensitivity and specificity [25]. One assay that shows promise is the TPTest, which detects *Salmonella* Typhi and *Salmonella* Paratyphi A antibody secretions by isolated lymphocytes [26, 27]. Most of the diagnostic assays developed to date target typhoidal *Salmonella*, but with additional modifications, many of these tests could be adapted for detection of iNTS.

Our goal here was to develop a quantitative real-time PCR (qPCR)–based methodology to detect *Salmonella* Typhi and *Salmonella* Paratyphi A directly from blood without the need for a preincubation step. We aimed to design an assay that would be more sensitive than blood culture but just as specific, and significantly faster in terms of time to detection.

MATERIALS AND METHODS

Bacterial Strains and Blood

Salmonella Typhi Ty2 and *Salmonella* Paratyphi A ATCC9150 were used for assay optimization. Additional clinical invasive *Salmonella enterica* strains (serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C, Typhimurium, Enteritidis, and Dublin) were previously obtained from blood or other sterile sites in Mali (Typhi and NTS) or Chile (Typhi, Paratyphi A, and Paratyphi B). Other bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica* serovars Choleraesuis and Newport) were from the Centers for Disease Control and Prevention or the Center for Vaccine Development culture collection. *Salmonella* species, *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were grown in HS bacteriological medium (5 g sodium chloride, 10 g soytone [Teknova, Hollister, California], 5 g Hy Yest 412 [Sigma Aldrich, St Louis, Missouri] in 1 L distilled water) at 37°C. *Streptococcus pneumoniae* and *H. influenza*e were subcultured on commercially available plates (BD BBL Trypticase Soy Agar with 5% Sheep Blood/Chocolate II, I Plate Prepared Media [Fisher Scientific, Pittsburgh, Pennsylvania]) at 37°C. Whole human blood with sodium heparin anticoagulant was purchased from Biological Specialty Corporation (Colmar, Pennsylvania) or from healthy donors from the Baltimore area under the approval of the University of Maryland, Baltimore Institutional Review Board. All blood donors provided informed written consent.

DNA Extraction

The white blood cell fraction (WBC) was isolated from ≤3 mL of whole blood using erythrocyte lysis buffer as previously described [28]. The supernatant was decanted and DNA extractions were performed using the QIAamp Blood DNA Mini kit

(Qiagen) on the WBC pellets with modifications to the manufacturer's protocol as described in the Supplementary Data. DNA was eluted in 32 µL of nuclease-free water.

Real-time PCR

Primers and probes used in this study are shown in Table 1. Probes were designed in Primer express version 2.0 (Applied Biosystems). Primers were designed using Primer3 version 4.0.0 [29, 30]. Real-time PCR was performed using a 20-µL reaction containing 10 µL TaqMan Fast Universal PCR Master Mix (2×) (Life Technologies, Grand Island, New York), 1 µL probeset mix (900 nM probe, 250 nM forward primer, and 250 nM reverse primer), and 9 µL template. The instrumentation used was the 7500 FAST Dx Real-time PCR machine (Life Technologies). Initial denaturation was at 95°C for 20 seconds followed by 50 cycles of 95°C denaturation for 3 seconds, and 60°C annealing and extension for 30 seconds. Data was collected during the annealing and extension step. A positive was determined if amplification intersected with the threshold within 50 cycles. Results reported as an undetermined cycle threshold (Ct) were examined for indications of late amplification as described in the Supplementary Data. Controls were run with every reaction as described in the Supplementary Data.

In Vitro Evaluation of Specificity

Specificity of primers was tested on clarified boiled lysate of *Salmonella enterica* (serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C, Typhimurium, Enteritidis, Dublin, Choleraesuis, and Newport) and other bacteria including *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, *P. aeruginosa*, and *E. coli* (Table 2). Three to 5 colonies were suspended in molecular-grade water and boiled at 95°C for 10 minutes in a thermal cycler. The lysate was centrifuged at maximum speed in a microcentrifuge for 1 minute. The supernatant was transferred into a fresh tube and 2 µL of a 1:10 dilution of the clarified lysate was tested in a 20-µL reaction for amplification of the *clyA* amplicon.

In Vitro Evaluation of Sensitivity

Salmonella Typhi reference strain Ty2 was grown overnight in HS media to approximately 1×10^9 CFU/mL. Serial 1:10 dilutions of bacteria were made in phosphate-buffered saline. A total of 100–200 µL of bacterial suspension was spiked onto the WBC pellet that was generated following erythrocyte lysis. Total genomic DNA was extracted as described above. Actual CFU used to spike WBC pellets was determined by performing viable counts using the dilutions expected to contain 100–1000 CFU/mL.

Table 1. Primers and Probes Used in This Study

Oligo	Sequence (5′ to 3′) and Fluorophore	Target	Reference
Cloning primers			
ST-Fc	GGAGTCGCCGTTTTTAGACA	*Salmonella* Typhi STY0201	This work
ST-Rc	TCCTTCAGCCAGCAGAGAAT	*Salmonella* Typhi STY0201	This work
PA-Fc	AATTGGCGGCGTAGTGATAG	*Salmonella* Paratyphi A SSPA2308	This work
PA-Rc	GTGAGGGGACAGATGTGGAG	*Salmonella* Paratyphi A SSPA2308	This work
clyA559-F	ATAGTCGCCGGTCCGTTTG	*Salmonella* Typhi *clyA*	This work
clyA722-R	GCCGCATCGATATCTTTATTCG	*Salmonella* Typhi *clyA*	This work
Diagnostic primers and probes			
ST-Probe	FAM-CATTTGTTCTGGAGCAGGCTGACGG-TAMRA	*Salmonella* Typhi STY0201	[22]
ST-Frt	CGCGAAGTCAGAGTCGACATAG	*Salmonella* Typhi STY0201	[22]
ST-Rrt	AAGACCTCAACGCCGATCAC	*Salmonella* Typhi STY0201	[22]
Pa-Probe	CY5-CCCATACAATTTCATTCTTATTGAGAATGCGC-BHQ2	*Salmonella* Paratyphi A SSPA2308	[22]
Pa-Frt	ACGATGATGACTGATTTATCGAAC	*Salmonella* Paratyphi A SSPA2308	[22]
Pa-Rrt	TGAAAAGATATCTCTCAGAGCTGG	*Salmonella* Paratyphi A SSPA2308	[22]
phHV-Probe	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2	Recombinant pCR TOPO 2.1 gB	[22]
PhHV-Frt	GGGCGAATCACAGATTGAATC	Recombinant pCR TOPO 2.1 gB	[22]
PhHV-Rrt	GCGGTTCCAAACGTACCAA	Recombinant pCR TOPO 2.1 gB	[22]
clyA542-Probe	FAM-CCGGTGCTGCAGCAGGCATA-TAMRA	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA498-F	TTATTTCCAGTCACAGGTGGATAG	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA677-R	CTAGTAAAGAAATTTTGCACTGCTTTTA	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA383-Probe	FAM-AACTGAATGAAGCGCAAAAATCTCTCCTGG-TAMRA	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA335-F	CAGCGCAGAAAGACATTCTCATC	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA440-R	GAAGCGTTGTTGAAACTTTGTGAA	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work
clyA598-Probe	FAM-ATTGCTGCGGGCGTGATTGAAGGGA-TAMRA	*Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*	This work

Table 2. Specificity of the *clyA* Probeset

Species	Serovar, Pathotype, or Strain Name (Source)	No. of Strains Tested	Positive or Negative by qPCR
Escherichia coli	BORT (CVD)	1	–
E. coli	EPEC E2348/69 (CVD)	1	+
E. coli	EAEC O42 (CVD)	1	+
Streptococcus pneumoniae	Serotypes 6b, 14, 19f, 23 (CVD)	4	–
Haemophilus influenzae	Strains 0183 and 0255 (CVD-Mali)	2	–
Klebsiella pneumoniae	B5055 (CVD)	1	–
Pseudomonas aeruginosa	PAO1 (CVD)	1	–
Salmonella enterica	Paratyphi B MNZ6203 (CVD-Chile)	1	–
S. enterica	Paratyphi C P53 (CVD-Mali)	1	–
S. enterica	Typhimurium SL13444 (CVD), I77 (CVD-Mali)	2	–
S. enterica	Enteritidis R11 (CVD-Mali)	1	–
S. enterica	Dublin P10, R17 (CVD-Mali)	2	–
S. enterica	Choleraesuis 06–0868 (CDC)	1	–
S. enterica	Newport 07-0044 (CDC)	1	–
S. enterica	Multiple *Salmonella* Typhi clinical strains (CVD-Mali and CVD-Chile)	24	+
S. enterica	Multiple *Salmonella* Paratyphi A clinical strains (CVD-Mali and CVD-Chile)	12	+

Abbreviations: CDC, Centers for Disease Control and Prevention; CVD, CVD culture collection; CVD-Chile, isolated in Chile; CVD-Mali, isolated in Mali; EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; qPCR, real-time polymerase chain reaction.

Study Sites

Evaluation of the *Salmonella* qPCR-based diagnostic was performed in Karachi, Pakistan. The study sites for the enrollment of typhoid cases included (1) the emergency department of the Aga Khan University (AKU) Hospital; (2) the main laboratory of the AKU Hospital; and (3) specimen collection point at Garden (AKU satellite laboratory). Controls were enrolled at AKU's Department of Paediatrics–run primary healthcare centers, situated in low-income areas of Karachi (Rehri Goth, Bhains Colony).

Participants

Participants with the following inclusion criteria participated in the study: 5–18 years old, clinically suspected typhoid or paratyphoid fever (enteric fever), with documented fever ≥38°C and no other identified focus of infection at the time of presentation, who provided consent and assent to obtain blood for culture and qPCR. Participants meeting any of the following criteria were excluded from study participation: fever with clinical signs indicating a clear focus of infection making a diagnosis of enteric fever unlikely; diagnosed cases of hematological malignancies presenting with febrile neutropenia; or refusal for consent or assent. Control participants who met the following inclusion criteria participated in the study: children aged 5–18 years with no signs of active infection, who provided consent and assent to obtain blood for culture and qPCR.

Clinical Study Laboratory Methods

Equal volumes of blood were tested by blood culture and real-time PCR in a blinded fashion. For children aged 5–14 years, up to 3 mL blood was tested by blood culture and an equivalent volume tested by qPCR. For children aged 14–18 years, up to 8 mL blood was tested by blood culture and an equivalent volume by qPCR. Blood culture was performed using a Bactec 9050 machine and standard microbiological techniques. Real-time PCR was performed by lysing erythrocytes, isolating DNA using a QIAamp DNA Blood Mini Kit (Qiagen) and then detecting *Salmonella* Typhi and *Salmonella* Paratyphi A by targeting the *clyA* gene as described above.

Statistical Analysis

Prism5 was used for most statistical analyses and graphical representation. Data were analyzed using Mann–Whitney test (2-tailed) or 1-way analysis of variance (ANOVA). Results were considered significant if $P < .05$. Sensitivity and specificity with 95% confidence intervals were calculated using 2×2 tables on the MedCalc.net website (https://www.medcalc.net/tests/diagnostic_test.php).

Research Ethics

This study was approved by the AKU Research Ethics Committee and the institutional review board of the University of Maryland School of Medicine.

RESULTS

Development of a Highly Sensitive qPCR Assay

We designed several primers and probes to detect simultaneously *Salmonella* Typhi and *Salmonella* Paratyphi A. We

targeted *clyA* (also known as *hlyE*), which is conserved in *Salmonella* Typhi and *Salmonella* Paratyphi A but absent from other *Salmonella* serovars [31, 32]. Certain *E. coli* also harbor *clyA* [33], so we attempted to design probesets that would be specific for *Salmonella* Typhi and *Salmonella* Paratyphi A *clyA*, but they were not as efficient as other probesets that also bound *E. coli* (data not shown). We assessed the efficiency of several *clyA* primers and probes and determined that clyA598-probe, and primers clyA559-F and clyA722-R were as efficient as previously described *Salmonella* Typhi and *Salmonella* Paratyphi A probesets [22] (Supplementary Table 1). This probeset was used for all subsequent analyses.

Next, we optimized preparation of DNA template to enhance sensitivity. We have previously shown that by targeting lymphocytes (either by lysing erythrocytes or by isolating the buffy coat), we are able to subsequently extract DNA using a mini DNA isolation kit instead of a larger DNA isolation kit [28]. We showed that the limit of qPCR detection was decreased when erythrocyte lysis was performed prior to DNA extraction using the QIAamp Blood DNA Mini kit (Qiagen) compared with when DNA was directly isolated using the QIAamp Blood DNA Midi kit (Qiagen). In the present study, we improved our limit of detection by modifying the DNA extraction procedure as described in the Supplementary Data. A schematic diagram of the DNA extraction and qPCR procedure is shown in Supplementary Figure 1.

We also enhanced sensitivity by testing as much of the available sample as possible. Other PCR-based methods only use a fraction of available DNA template for amplification [22]. Here, we eluted DNA in 32 µL nuclease-free water and tested the majority of the sample by qPCR (3 reactions containing 9 µL of template each). We hypothesized that if there was 1 bacterium present in 3 mL blood, which is likely to occur based on results from previous studies [14], when this sample is tested by qPCR, the *clyA* gene target could conceivably be pipetted into a single well. As such, we interpreted a sample as being positive for qPCR if at least 1 well was positive. To maximize the chances of detecting a positive result, we used a Ct cutoff of 50 instead of 40 as is generally used for other assays. We examined the qPCR raw data closely to ensure that wells that were interpreted as positive exhibited true amplification as described in the Supplementary Data.

In Vitro Sensitivity and Specificity Using Spiked Blood

We evaluated the optimized DNA extraction and qPCR methodology in the laboratory and determined in vitro sensitivity and specificity. We isolated lymphocytes from 2–3 mL blood and then spiked with various concentrations of *Salmonella* Typhi, isolated DNA, and performed qPCR using the *clyA* probeset. As shown in Figure 1, 2 separate users were able to detect approximately 1 CFU/mL blood and even detect as few as

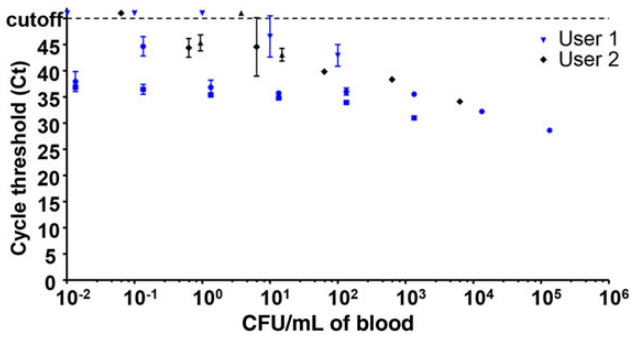

Figure 1. In vitro sensitivity of the *Salmonella* real-time polymerase chain reaction (qPCR) assay. Lymphocytes were isolated from 2–3 mL whole human blood by lysing red blood cells. Cell pellets were spiked with various concentrations of *Salmonella* Typhi Ty2, and DNA was isolated using a QIAamp Blood DNA Mini kit and tested using the *clyA* qPCR. The colored symbols represent 2 different users and the various symbols represent experiments performed on different occasions. Data are expressed as mean ± standard deviation from 3 replica wells. The maximum cycle threshold (Ct) value is 50 cycles. Wells with an undetermined Ct were assigned a Ct of 51.

0.01 CFU/mL (most likely detecting dead bacteria). As expected, we observed a dose-response curve whereby higher concentrations of *Salmonella* Typhi yielded lower Ct values. We observed this dose-response curve for higher concentrations of bacteria (>10 CFU/mL) but not for lower concentrations (<10 CFU/mL). We believe that at concentrations <10 CFU/mL, sampling effects are occurring and one either observes detection (obtain a Ct value) or no detection (expressed by the machine as undetermined but which we have expressed as a Ct of 51 in Figure 1). Even in conditions with near-perfect reaction efficiency [34], consistent replicates for Ct are obtained when there are >10 copies of target [35], but variation in Ct is greater for low copy targets [36]. Spiking of blood with <0.01 *Salmonella* Typhi CFU/mL did not result in amplification.

We also determined whether the *clyA* probeset could detect other *Salmonella* serovars or any non-*Salmonella* bacteria. As expected, enteroaggregative *E. coli* and enteropathogenic *E. coli* were detected (Table 2). However, *E. coli* Bort, which is an invasive *E. coli* strain, was negative by qPCR. We confirmed that the probeset could detect 24 *Salmonella* Typhi and 12 *Salmonella* Paratyphi A strains. All of the other *Salmonella* serovars (Typhimurium, Enteritidis, Dublin, Paratyphi B, Paratyphi C, Choleraesuis, Newport) and non-*Salmonella* strains (*K. pneumoniae*, *S. pneumoniae*, *H. influenzae*, *P. aeruginosa*) tested negative by qPCR.

Evaluation of Specificity of the qPCR Diagnostic Using Healthy US Donors

We determined the specificity of our *clyA* qPCR-based diagnostic assay by testing blood from 97 healthy US donors. Whole

Table 3. Specificity of the *clyA* Probeset Using Blood From Healthy US Donors

Positive or Negative by qPCR	No. of Volunteers
Negative	93 (96%)
Positive	4 (4%)
Total	97

Abbreviation: qPCR, real-time polymerase chain reaction.

human blood was lysed using erythrocyte lysis buffer and DNA was extracted from lymphocytes and tested by qPCR. Four specimens (4%) tested positive by qPCR (Table 3). For each these 4 specimens, only 1 well tested positive (out of 3) and produced high Ct values of 38.8, 38.93, 41.54, and 46.78. Therefore, using blood from healthy US donors, the specificity of the qPCR assay is 96%.

Evaluation of the qPCR Diagnostic in a Typhoid-Endemic Region

We evaluated our qPCR-based diagnostic in a pediatric population in Karachi, Pakistan. We enrolled 136 children (5–18 years old) who had fever (≥38°C) for at least 3 days (cases) and 118 healthy controls. An equal volume of blood was tested by blood culture and qPCR. Twenty children tested positive for *Salmonella* Typhi or *Salmonella* Paratyphi A using standard microbiological methods (Table 4). Of these, 14 possessed *Salmonella* Typhi and 6 possessed *Salmonella* Paratyphi A. An additional 4 children tested positive for *Bacillus* species (2 cases), *Micrococcus* species (1 case), and *Staphylococcus* species (1 case). Of the 20 cases that were blood culture positive for *Salmonella* Typhi or *Salmonella* Paratyphi A, only 8 were also positive by qPCR. We confirmed that the *clyA* probeset was able to bind to DNA from all of the *Salmonella* Typhi and *Salmonella* Paratyphi A strains identified by blood culture (Table 5). Therefore, absence of detection was not due to absence of binding of primers or probe.

We determined sensitivity and specificity of the *clyA* qPCR and blood culture using each other as the comparator (Table 6). The *clyA* qPCR diagnostic was 40% as sensitive as blood culture.

Table 5. Bacteria Detected From Positive Blood Cultures of Cases in Karachi, Pakistan

Bacteria	No. of Strains	Confirmed to Bind to *clyA* Probeset
Salmonella Typhi	14	14
Salmonella Paratyphi A	6	6
Bacillus spp	2	ND
Micrococcus spp	1	ND
Staphylococcus spp	1	ND

Abbreviation: ND, not determined.

When qPCR-positive specimens were considered to be true positives, blood culture only exhibited 28.57% sensitivity. Specificity was ≥91%.

We examined the qPCR-positive specimens further to determine whether there were any differences between blood culture–positive and blood culture–negative specimens. We observed a lower Ct for blood culture–positive specimens than blood culture–negative specimens (mean ± standard deviation [SD], 39.89 ± 3.51 Ct vs 41.72 ± 5.13 Ct) but the difference was not significant ($P = .1335$, Mann–Whitney test; Figure 2). We also examined the ages of all of the children (cases and controls) that were either blood culture positive or qPCR positive or both (Figure 3*A*). There were no significant differences in age by 1-way ANOVA. Likewise, we also examined blood volumes tested by blood culture and qPCR for all blood culture–positive or qPCR-positive samples (Figure 3*B*). There were no significant differences in volumes tested by blood culture vs qPCR (blood culture negative/qPCR positive: mean ± SD, 3.29 ± 1.69 mL and 4.01 ± 1.94 mL, respectively; blood culture positive/qPCR negative: 4.03 ± 2.08 mL and 4.07 ± 1.64 mL, respectively; blood culture positive/qPCR positive: 4.21 ± 2.21 and 3.89 ± 2.05 mL, respectively; Mann–Whitney test). However, the blood culture–negative and qPCR-positive specimens were almost significantly different ($P = .0578$).

One major attribute that a new typhoid diagnostic should possess is a reduced time to detection and identification

Table 4. Blood Culture and Real-time Polymerase Chain Reaction Positivity for Cases and Controls in Karachi, Pakistan

Category	qPCR Positive	qPCR Negative	Total
Clinically diagnosed with enteric fever (cases)	23	113	136
Blood culture positive for *Salmonella* Typhi or *Salmonella* Paratyphi A	8	12	20
Blood culture negative (no bacteria detected)	15	101	116
Healthy controls	5	113	118
Blood culture positive for *Salmonella* Typhi or *Salmonella* Paratyphi A	0	0	0
Blood culture negative (no bacteria detected)	5	113	118

Abbreviation: qPCR, real-time polymerase chain reaction.

Table 6. Sensitivity and Specificity for Blood Culture and Real-time Polymerase Chain Reaction Assays Compared to Each Other Using Cases and Controls From Karachi, Pakistan

Test Assay	Comparator (True Positive)	Sensitivity (95% CI)	Specificity (95% CI)
qPCR	Blood culture	40% (19.12%–63.95%)	91.45% (87.11%–94.7%)
Blood culture	qPCR	28.57% (13.22%–48.67%)	94.69% (90.91%–97.23%)

Abbreviations: CI, confidence interval; qPCR, real-time polymerase chain reaction.

compared to blood culture. We measured the time taken to process blood samples by qPCR (erythrocyte lysis and DNA extraction and qPCR) compared with blood culture (detection by instrumentation and identification by classical clinical microbiology). As shown in Figure 4, qPCR is significantly faster (mean ± SD, 2 hours 34 minutes ± 15 minutes) than blood culture (mean ± SD, 37 hours 40 minutes ± 17 hours) in terms of detection and identification of *Salmonella* Typhi and *Salmonella* Paratyphi A ($P < .0001$; Mann–Whitney test, 2-tailed).

DISCUSSION

Evaluation of diagnostic tests for invasive *Salmonella* infection is particularly challenging in light of the fact that the "gold standard" comparator diagnostic in most field trials is blood culture, which exhibits low sensitivity itself. Although we observed a higher sensitivity for detection by qPCR than blood culture, we did not detect bacteria by qPCR in 12 of 20 blood culture–positive specimens. A possible explanation is that due to sampling effects, *Salmonella* Typhi and *Salmonella* Paratyphi A in these blood samples were aliquoted into the blood culture bottles but not the tubes tested by qPCR. The average volume of blood tested in these samples was approximately 4 mL and the median concentration of *Salmonella* Typhi in blood is 0.3–1 CFU/mL, which means that there was 1.2–4 CFU per sample [14, 15]. Therefore, it is conceivable that for the blood culture–positive/qPCR-negative specimens, 1 CFU was detected by blood culture, but due to sampling, there were no bacteria available for detection by qPCR.

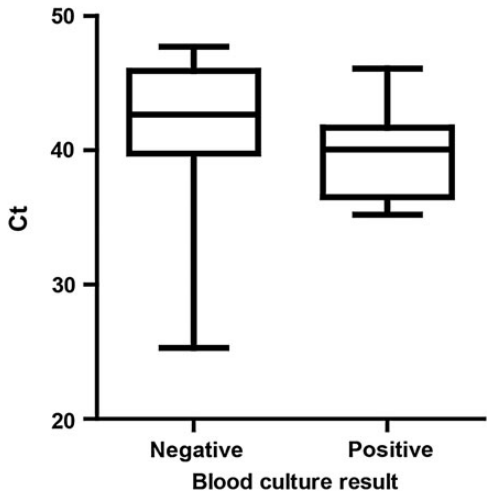

Figure 2. Cycle threshold (Ct) values of real-time polymerase chain reaction–positive specimens according to blood culture result. Data are expressed as a box-and-whisker plot with whiskers representing the minimum and maximum value.

Our data suggest that the blood culture–negative but qPCR-positive specimens had a lower bacterial concentration (than the blood culture–positive specimens), which the qPCR-based assay was able to detect but which could not be cultured. However, the differences in Ct values were not statistically significant. Based on our results, we recommend that future field trials employ multiple diagnostic methodologies to define a "positive" sample. We suggest that future typhoid/paratyphoid diagnostic assays should first be tested in an adult population where larger blood volumes can be obtained and tested simultaneously in various assays. We propose that new *Salmonella* diagnostic assays be evaluated in clinical studies with various comparators. If possible, the novel diagnostic should be compared to bone marrow culture as this method has shown the highest sensitivity to date. If bone marrow culture is not feasible, then blood culture should be performed with at least 2 other diagnostic tests (eg, qPCR with or without preenrichment vs an immunoassay such as the TPTest). If at least 2 assays test positive, then the specimen should be considered a true positive. Once proof of principle has been established, then the assay should be validated in adults from other geographic regions and in pediatric populations.

For iNTS diagnostics, a similar approach could be used as for typhoid diagnostics but with slight differences due to the populations that are susceptible to iNTS infections. The iNTS assays could first be tested in human immunodeficiency virus–infected adults and then evaluated in infants. These studies would have to be performed in Africa.

In addition to standard quantitative metrics of performance, operational characteristics of future typhoid and iNTS assays must be considered as well. These include factors such as time to definitive diagnosis, technical simplicity, cost and stability of reagents and equipment, and level of staff training needed to reliably perform a test and interpret the results. There are

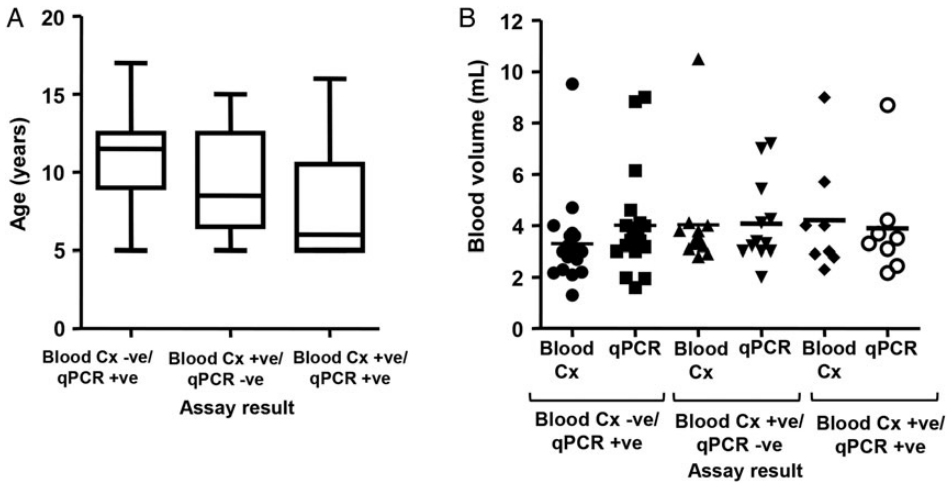

Figure 3. Age (*A*) and volume of blood tested (*B*) of specimens that were blood culture negative and real-time polymerase chain reaction (qPCR) positive; blood culture positive and qPCR negative; and positive for both blood culture and qPCR. Age data are expressed as a box-and-whisker plot with whiskers representing the minimum and maximum value. Blood volumes are expressed as a scatter plot with the bar indicating the mean. Abbreviations: -ve, negative; +ve, positive; Cx, culture.

many advantages of PCR over other technologies, including the ability to detect various bacterial targets on the same platform/equipment; permit speciation, detection of antibiotic resistance genes, and evolution of target selection as knowledge of circulating outbreak strains evolves; and lack of necessity for bacterial viability. However, there are several disadvantages to PCR, including that the high degree of specificity can limit the utility of specific primer sets to validated target species and requires limit of detection, sensitivity, and specificity testing on many serovars [37]; targets must be selected that are present and retained across relevant species and serovars; a centralized laboratory with skilled personnel is required [10]; and it is susceptible to contamination.

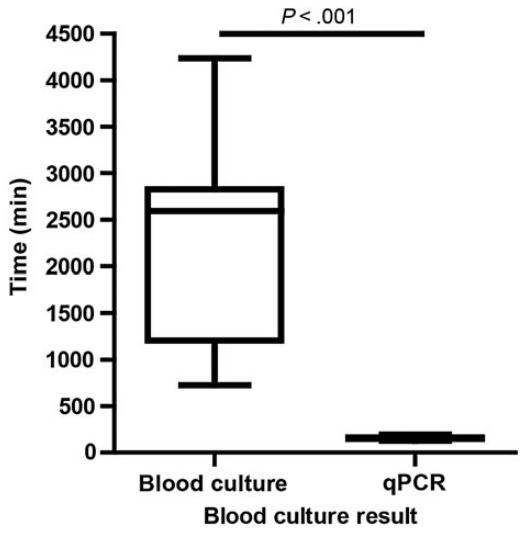

Figure 4. Time to identification for blood culture or real-time polymerase chain reaction (qPCR) positivity. Data are expressed as a box-and-whisker plot with whiskers representing the minimum and maximum value. Data were analyzed using Mann–Whitney test, 2-tailed.

A significant strength of this study is that the entirety of the assay (including nucleic acid extraction, qPCR, and analysis) was performed on-site, in a relevant hospital laboratory, by intended end-user technicians. Although test optimization and validation in the laboratory environment are critical preliminary steps, performance characteristics must subsequently be evaluated in the field setting for which the assay is intended [38]. Discrepancies in performance characteristics between use in developing laboratories and field testing is particularly important for assays with increased sensitivity or technical complexity (such as nucleic acid detection tests), as numerous factors including reagent transport conditions and equipment or operator variability can substantially alter performance.

Two desirable attributes of a typhoid diagnostic are improved sensitivity compared to blood culture and reduced time to detection. Here, we clearly show that real-time PCR is significantly faster at detecting and identifying *Salmonella* Typhi or *Salmonella* Paratyphi A than classical microbiological techniques. We would expect the same to also be true for detection of iNTS.

Development of new and improved typhoid and paratyphoid diagnostics has been challenging, but with further testing and evaluation, diagnostic assays that are suitable for use in developing countries are on the horizon [39]. The first *Salmonella* diagnostics should target *Salmonella* Typhi and *Salmonella* Paratyphi A in preparation for imminent typhoid and paratyphoid

vaccine evaluation programs [40, 41]. However, additional diagnostic assays should also target iNTS such as *Salmonella* Typhimurium and *Salmonella* Enteritidis, which are a significant cause of morbidity and mortality in sub-Saharan Africa and for which vaccines are also in development [4, 41].

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Dr Stephen Baker for the kind gift of the PhHV-1 *g*B plasmid. We also thank Dr Sandra Panchalingam for suggestions made about real-time polymerase chain reaction (qPCR) analysis and interpretation.

Financial support. This work was supported by the Bill & Melinda Gates Foundation (grant number OPP1039437 to M. M. L.).

Supplement sponsorship. This article appeared as part of the supplement "Invasive *Salmonella* Disease in Africa," sponsored by the University of Otago.

Potential conflicts of interest. During this work, A. K. Z. joined the Bill & Melinda Gates Foundation. To avoid potential conflicts of interest, F. Q. replaced A. K. Z. as the AKU Principal Investigator. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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