Identification by PCR of Non-typhoidal *Salmonella enterica* Serovars Associated with Invasive Infections among Febrile Patients in Mali

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**Abstract**

**Background:** In sub-Saharan Africa, non-typhoidal *Salmonella* (NTS) are emerging as a prominent cause of invasive disease (bacteremia and focal infections such as meningitis) in infants and young children. Importantly, including data from Mali, three serovars, *Salmonella enterica* serovar Typhimurium, *Salmonella* Enteritidis and *Salmonella* Dublin, account for the majority of non-typhoidal *Salmonella* isolated from these patients.

**Methods:** We have extended a previously developed series of polymerase chain reactions (PCRs) based on O serogrouping and H typing to identify *Salmonella* Typhimurium and variants (mostly I 4,[5],12:i:-), *Salmonella* Enteritidis and *Salmonella* Dublin. We also designed primers to detect *Salmonella* Stanleyville, a serovar found in West Africa. Another PCR was used to differentiate diphasic *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium from other O serogroup B, H1 serovars. We used these PCRs to blind-test 327 *Salmonella* serogroup B and D isolates that were obtained from the blood cultures of febrile patients in Bamako, Mali.

**Principal Findings:** We have shown that when used in conjunction with our previously described O-serogrouping PCR, our PCRs are 100% sensitive and specific in identifying *Salmonella* Typhimurium and variants, *Salmonella* Enteritidis, *Salmonella* Dublin and *Salmonella* Stanleyville. When we attempted to differentiate 171 *Salmonella* Typhimurium (I 4,[5],12:i:1,2) strains from 52 monophasic *Salmonella* Typhimurium (I 4,[5],12:i:-) strains, we were able to correctly identify 170 of the *Salmonella* Typhimurium and 51 of the *Salmonella* 1,i:12:i:- strains.

**Conclusion:** We have described a simple yet effective PCR method to support surveillance of the incidence of invasive disease caused by NTS in developing countries.

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**Introduction**

In industrialized countries, non-typhoidal *Salmonella* (NTS) constitute a well recognized public health problem that in healthy subjects is overwhelmingly encountered clinically as self-limited gastroenteritis [1,2]. In immunocompromised and debilitated hosts, NTS can become invasive, leading to bacteremia, sepsis and focal infections (e.g., meningitis) [2,3]. Among infants less than three months of age who become infected with NTS in industrialized countries, invasiveness is also occasionally observed, resulting in bacteremia and focal infections [4].

Interestingly, whereas systematic blood culture-based surveillance of febrile pediatric patients in Asia has clearly highlighted the high incidence of bacteremia associated with *Salmonella enterica* serovars Typhi and Paratyphi A in children residing in crowded urban settings [5–7], isolation of NTS has not been common. In striking contrast, systematic blood culture-based surveillance and clinical studies of hospitalized and ambulatory pediatric patients <60 months of age with fever or focal infections in sub-Saharan Africa have documented the important role of NTS as invasive bacterial pathogens [8–17]. NTS constituted one of the three most common invasive bacterial pathogens in all these studies.
Author Summary

The genus *Salmonella* has more than 2500 serological variants (serovars), such as *Salmonella enterica* serovar Typhi and *Salmonella* Paratyphi A and B, that cause, respectively, typhoid and paratyphoid fevers (enteric fevers), and a large number of non-typhoidal *Salmonella* (NTS) serovars that cause gastroenteritis in healthy hosts. In young infants, the elderly and immunocompromised hosts, NTS can cause severe, fatal invasive disease. Multiple studies of pediatric patients in sub-Saharan Africa have documented the important role of NTS, in particular *Salmonella* Typhimurium and *Salmonella* Enteritidis (and to a lesser degree *Salmonella* Dublin), as invasive bacterial pathogens. *Salmonella* spp. are isolated from blood and identified by standard microbiological techniques and the serovar is ascertained by agglutination with commercial antisera. PCR-based typing techniques are becoming increasingly popular in developing countries, in part because high quality typing sera are difficult to obtain and expensive and H serotyping is technically difficult. We have developed a series of polymerase chain reactions (PCRs) to identify *Salmonella* Typhimurium and variants, *Salmonella* Enteritidis and *Salmonella* Dublin. We successfully identified 327 *Salmonella* isolates using our multiplex PCR. We also designed primers to detect *Salmonella* Stanleyville, a serovar found in West Africa. Another PCR generally differentiated diphasic *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium variant strains from other closely related strains. The PCRs described here will enable more laboratories in developing countries to serotype NTS that have been isolated from blood.

Importantly, two serovars, *Salmonella* Typhimurium (and Typhimurium variants) and *Salmonella* Enteritidis have been reported to account for 79–95% of all bacteremic non-typhoidal *Salmonella* infections in sub-Saharan Africa [9,11–13,15,16,18,19]. *Salmonella* Dublin has been associated with a few percent of cases in some studies [12,13] but with a more substantial proportion in Mali [18], where a fourth serovar, *Salmonella* Stanleyville, also accounted for a notable proportion of all isolates [18], bringing the cumulative total to >95% of all strains.

We previously developed a multiplex polymerase chain reaction (PCR)-based approach to identify the three main pathogens responsible for typhoid (*Salmonella Typhi*) and paratyphoid (*Salmonella Paratyphi A* and *Salmonella Paratyphi B*) fevers [18]. Three sequential PCRs identify strains of *Salmonella* serogroups A, B or D (and Vi positive or negative); strains that express Phase 1 flagellar (H) antigen types H:a, H:b or H:d; and strains incapable of fermenting d-tartrate (d-T). By means of this PCR technology, *Salmonella Typhi* (O serogroup D, Vi+; H:a), *Salmonella Paratyphi A* (O serogroup A; H:a) and *Salmonella Paratyphi B* (O serogroup B; H:b; d-T non-fermenter) strains were identified with 100% sensitivity and 100% specificity.

Classical *Salmonella* serotyping methods identified the serovars of 336 NTS isolates from blood cultures of febrile children <16 years of age in Bamako, Mali, obtained in the course of systematic surveillance of children admitted to hospital or seen in the Emergency Department with fever or invasive infection syndromes [20–22]. *Salmonella* Typhimurium and “variants” (mainly I 4,5,[5],12:i:-), *Salmonella* Dublin, *Salmonella* Enteritidis and *Salmonella* Stanleyville were the most commonly isolated NTS [18]. Herein, we describe PCRs that when used in conjunction with the O serogrouping PCR described by Levy et al. [18] can identify *Salmonella* Typhimurium and variants (O serogroup B; H:i), *Salmonella* Enteritidis (O serogroup D, Vi+; H:g,m), *Salmonella* Dublin (O serogroup D, Vi+ or Vi--; H:g,p) and *Salmonella* Stanleyville (O serogroup B; H:a2,a23) with 100% sensitivity and 100% specificity. We anticipate that this methodology will be useful in reference laboratories and major clinical microbiology laboratories to identify *Salmonella* isolated from blood and other sterile sites in developing countries where robust PCR-based typing techniques are becoming increasingly popular and because high quality H typing sera are difficult to obtain, expensive and technically demanding to use.

Methods

Ethics statement

The surveillance protocol and consent form were reviewed by the Ethics Committee of the Faculté de Médecine, Pharmacie et Odonto-Stomatologie, Université de Bamako, and by the Institutional Review Board of the University of Maryland, Baltimore. For any patient eligible for laboratory surveillance to detect invasive bacterial disease, informed consent was obtained prior to their enrollment; ~95% of eligible subjects agreed to participate. Since the literacy rate in Bamako is <30%, as is customary practice for CVD-Mali clinical studies [20–22], the consent form was translated into Bambara and several other local languages and the translations recorded on audiotope [20]. CVD-Mali personnel explain the study, including the objectives and risks and benefits associated with participation. The audiotaped version of the consent form is then played and any questions posed are answered. Once the parent or patient has had all questions answered and agrees to participate, this is documented on a printed consent form written in French. If the participant is illiterate, a witness who is present throughout the consent procedure completes the necessary portions and signs the consent form; the parent/participant marks the consent form (either fingerprint or other notation). If the person is literate, then he/she may read and sign the consent form. This standard method of obtaining consent practiced by CVD-Mali was approved by ethics committees in Mali and at the University of Maryland.

Systematic surveillance for invasive bacterial infections

Since July 2002, clinical staff of the Centre pour le Développement des Vaccins du Mali (CVD-Mali) and the Hôpital Gabriel Touré (HGT) have been conducting systematic surveillance to detect invasive bacterial disease among hospitalized children <16 years of age [20–22]. Age-eligible children presenting to the emergency department with fever (≥39°C) or focal clinical findings suggestive of invasive bacterial infection (meningitis, septic arthritis, etc.) and requiring hospitalization are referred to CVD-Mali staff by the evaluating clinicians. A CVD-Mali physician obtains informed consent, records clinical and epidemiologic data, and obtains blood (and other relevant fluids) for culture in the HGT Clinical Bacteriology Laboratory. The child’s clinician is promptly notified when a culture yields a bacterial pathogen.

*Salmonella* strains

*Salmonella* Typhimurium strain 81.23500, *Salmonella* Enteritidis strain CVD SE and *Salmonella* Dublin strain 06-0707 were used to develop the multiplex PCR. Twenty-four control strains which came from the *Salmonella* Reference Laboratory of the Centers for Disease Control and Prevention (CDC), Atlanta, GA or the Center for Vaccine Development, Baltimore, MD have previously been described [18]. These strains were *Salmonella* serovars of various O serogroups (B, C1, C2, D, E1, O28 and O38) and H types (b, c, d, h, i, g, k, l, m, p, s, t, v, y, z10 and z29). Nine O serogroup B, Phase 1 flagella antigen H:i reference strains from the CDC were used to...
develop a PCR that discriminates between *Salmonella* Typhimurium and *I* 4,[5],12:i:- (Table 1). The NTS test strains consist of 327 *Salmonella* serogroup B and D isolates that were originally obtained from the blood cultures of febrile patients at l’Hôpital Gabriel Touré in Bamako, Mali. These strains were identified by conventional microbiological and classical serotyping methods by the CVD and CDC, as previously described [18]; 69 isolates were O serogroup D, including 37 *Salmonella* Enteritidis and 32 *Salmonella* Enteridiensis, and 250 isolates were O serogroup B.

**Primer Development**

**Detection of *Salmonella* Typhimurium and variants, *Salmonella* Enteritidis and *Salmonella* Dublin (and later *Salmonella* Stanleyville).** This primer mix contained the following primers: H-for, a primer sequence common to *B*, *H:i* serovars. To accomplish this, we used a primer mix and the monophasic serovar I 4,[5],12:i:- and other O serogroup discriminates between O serogroup D, including 37 *Salmonella* Enteritidis and 258 isolates were O serogroup B.

**Table 1. Nine reference strains of *Salmonella* consisting of serovars that belong to O group B and that possess Phase 1 H flagella antigen "i".**

| Strain | Serovar | O antigens | Phase 2 H flagella antigen(s) |
|--------|---------|------------|-------------------------------|
| CDC 443 | Gloucester | 1,4,12,(27) | 1w |
| CDC 513 | Agama | 4,12 | 1,6 |
| CDC 1045 | Lagos | 1,4,12 | 1,5 |
| CDC 1638 | Tsevie | 4,12 | e,n,z,15 |
| CDC 1855 | Lagos | 1,4,12 | 1,5 |
| CDC 2322 | Farsta | 4,12 | e,n,x |
| CDC 2419 | Turnod | 1,4,12 | z6 |
| CDC 07-0794 | 1,4,5,12:i:- | 4,5,12 | – |
| CDC 07-0972 | 1,4,5,12:i:- | 4,5,12 | – |

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**Table 2. Primers used in this study.**

| Primer | Sequence (5’ to 3’) | Amplicon (bp) | Reference |
|--------|---------------------|---------------|-----------|
| H-for | ACTCAGGCTTCCCGTAAAGGC | 779 | [18] |
| Hgp | ATAAATATCAGGCGCGCCGAA | 779 | This study |
| HI | ATAGCACTTTACCCGTC | 551 | [37] |
| Hz4,z23F | TTTGCAAGATGTCTACGCGC | 427 | This study |
| Hz4,z23R | AGGTAGTATGCGACGATCT | This study |
| sdlF | TGGTTTTATCTGATCGAACAGAG | 333 | [27] |
| sdlR | CGTCCTTGACTACGATGAC | [27] |
| 16SF | AATACGTCCCGGCGCTTG | 167 | Based on universal bacterial 16S rRNA gene primers that were included to ensure that DNA was added (Table 2). |
| DG74 | AGGAGGTGATCCAACCGCA | [47] |
| Sense-59 | CAACACAAACCTGGAGCGGTGCG | 1389 | [26] |
| Antisense-83 | GCCATATATTACCGCTTCCGCCG | [26] |
| FFLIB | CTGGCGACGAGTCTGCGATG | 250 or 1000 | [24] |
| RFLIA | GCGGTATACAGTGAATTCAC | [24] |

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**Results**

A multiplex PCR to detect *Salmonella* Typhimurium and monophasic variants, *Salmonella* Enteritidis and *Salmonella* Dublin

Figure 1 shows that the primers within the multiplex PCR were able to clearly identify the appropriate NTS alleles. A 779-bp containing primers FFLIB and RFLIA, which amplify the *fliB-fliA* intergenic region, and primers Sense-59 and Antisense-83, which amplify the Phase 2 (*fliB*) flagellar gene.

**DNA extraction and PCR**

PCR was performed in 1× PCR buffer, 3.5 mM MgCl₂, 0.2 mM of dNTPs and 0.2 U of Invitrogen Taq DNA polymerase (final volume of 25 μl) in an Eppendorf Mastercycler®. The primer mixes contained primers at a concentration of 5 μM each (final concentration of 0.2 μM) except for FFLIB and RFLIA that were used at a concentration of 10 μM each and the positive control primers (16SF and DG74) that were used at a concentration of 2.5 μM each. For each PCR reaction, 1.0 μl of primer mix was used. Crude DNA was prepared by suspending 3 colonies in 100 μl water and boiling for 10 min followed by centrifugation at 16,000×g for 30 sec and purified DNA was prepared using a GNOME DNA kit (QBIogene, Irvine, CA) according to the manufacturer’s instructions, and 5 μl of DNA was used in each PCR. The cycling parameters of the multiplex PCR that detects *H:i*, *H:gp* and *SdfI* involved denaturation at 95°C for 2 min, followed by 25 cycles comprised of heating to 95°C for 30 sec, 64°C for 30 sec and 72°C for 15 sec, and a final step of 72°C for 5 min. The cycling parameters of the PCR that discriminates between *Salmonella* Typhimurium and *I* 4,[5],12:i:- involved denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 sec, 64°C for 30 sec and 72°C for 1.5 min, and a final step of 72°C for 5 min. PCR products were separated on 2% (w/v) agarose gels, stained with ethidium bromide and visualized using a UV transilluminator.

**Table 2. Primers used in this study.**

- **H-for** ACTCAGGCTTCCCGTAAAGGC [18]
- **Hgp** ATAAATATCAGGCGCGCCGAA This study
- **HI** ATAGCACTTTACCCGTC 551 [37]
- **Hz4,z23F** TTTGCAAGATGTCTACGCGC 427 This study
- **Hz4,z23R** AGGTAGTATGCGACGATCT This study
- **sdlF** TGGTTTTATCTGATCGAACAGAG 333 [27]
- **sdlR** CGTCCTTGACTACGATGAC [27]
- **16SF** AATACGTCCCGGCGCTTG 167 Based on universal bacterial 16S rRNA gene primers that were included to ensure that DNA was added (Table 2).
- **DG74** AGGAGGTGATCCAACCGCA [47]
- **Sense-59** CAACACAAACCTGGAGCGGTGCG 1389 [26]
- **Antisense-83** GCCATATATTACCGCTTCCGCCG [26]
- **FFLIB** CTGGCGACGAGTCTGCGATG 250 or 1000 [24]
- **RFLIA** GCGGTATACAGTGAATTCAC [24]
A 551-bp product was amplified from *Salmonella* *Dublin* (*fliC*-*gp*), a 333-bp product was amplified from *Salmonella* *Enteritidis* (Sdf I). The internal positive control primers (universal 16S rRNA gene primers) amplified a 167-bp product from each strain.

**Initial validation of the multiplex PCR assay**

To preliminarily assess the specificity of the multiplex PCR assay, we tested 24 control *Salmonella* strains consisting of a range of serovars (previously described in [18]) in a blinded fashion (Figure 2). The multiplex PCR correctly identified *Salmonella Typhimurium* and *Salmonella Cotham* as H:i, *Salmonella Dublin* as H:g,p and *Salmonella Enteritidis* as containing Sdf I (Figure 2). Faint products of the size of Sdf I were observed for *Salmonella Meleagridis* and *Salmonella Livingstone*. However, *Salmonella Meleagridis* is O serogroup E1 and *Salmonella Livingstone* is O serogroup C1, so when also tested by our previously described O serogrouping PCR [10], these serovars would not be mistaken as *Salmonella Enteritidis*. The same is true for *Salmonella Cotham*, which although it possesses fliC-i, is not O serogroup B and would not be mistaken as *Salmonella Typhimurium*. Therefore, the new multiplex PCR was sensitive in terms of its ability to identify serovar Cotham as H:i and was specific, when combined with the O-serogrouping PCR, in showing that the strain was not serovar Typhimurium. We also blind-tested a sample of *Salmonella* *Typhi* and *Salmonella Paratyphi A* and B strains to ensure that the PCR would not detect these strains. The multiplex PCR correctly identified fliC-i of six *Salmonella Typhimurium*, Sdf I of four *Salmonella Enteritidis*, and fliC-g,p of five *Salmonella Dublin* strains but only the 16S rRNA gene was amplified from five strains each of serovars Typhi, Paratyphi A and Paratyphi B (data not shown).

**Analysis of 327 Salmonella serogroup B and D clinical isolates from Mali**

We blind-tested 69 non-Typhi serogroup D *Salmonella* and 258 serogroup B strains that were originally obtained from the blood cultures of febrile patients at l’Hôpital Gabriel Touré in Bamako,
Mali [18] with the multiplex PCR designed to identify *Salmonella Typhimurium* (I 4,[5],12:i:-) and variants (monophagic I 4,[5],12:i:- and non-motile (NM) I 4,[5],12:NM), *Salmonella Enteritidis* and *Salmonella Dublin*. This PCR was performed in parallel to serotyping. We correctly identified all the serogroup D isolates (37 *Salmonella Dublin* and 32 *Salmonella Enteritidis*) and all 232 *Salmonella Typhimurium* and variant strains (Table 3). If the *Salmonella* Typhimurium-like strains (i.e., I 4,[5],12:i:- and I 4,[5],12:NM) are included in the target group then the PCR is 100% sensitive and 100% specific in identifying *Salmonella Typhimurium*, *Salmonella Enteritidis*, *Salmonella Dublin* and *Salmonella* Typhimurium-like organisms. The remaining 26 serogroup B isolates were negative for the tested targets.

**Discrimination between *Salmonella Typhimurium* and I 4,[5],12:i:-**

During the course of this study, we decided to determine the prevalence of I 4,[5],12:i:- in Mali. Levy et al. [18] identified 220 *Salmonella Typhimurium*, four I 4,[5],12:i:- and eight I 4,[5],12:NM strains. However, in this previous study, Phase 2 flagella typing was not performed on all of the strains. We re-examined the 220 *Salmonella* O serogroup B, H:i isolates that had been previously been presumptively identified as *Salmonella Typhimurium* and used classical methods (i.e., sera against the Phase 2 H1,2 flagella) to determine that 48 isolates were in fact I 4,[5],12:i:- (bringing the total number of isolates of this serovar to 52) and one isolate was I 4,[5],12:NM (bringing the total number of isolates of this serovar to nine). The remaining 171 strains were confirmed as *Salmonella Typhimurium*.

We have combined previously described primers in a PCR to discriminate between *Salmonella Typhimurium* and I 4,[5],12:i:-. Primers FFLIB and RFLIA amplify the *flbD-flaA* intergenic region of the flagellin gene cluster [24]. *Salmonella Typhimurium* strains possess an IS200 fragment in this region [25]. Burnens et al. [25] showed that 21 of 23 isolates of *Salmonella Typhimurium* and none of 85 isolates of *Salmonella* serovars contained IS200 in this region. Primers FFLIB and RFLIA have been reported to amplify a 1-kb product from *Salmonella Typhimurium* and I 4,[5],12:i:- strains and a 250-bp product from all other serovars [24]. However, when validating these primers, we found that a 1-kb fragment was amplified from *Salmonella Farsta* (not tested by Echeita et al. [24]) suggesting that this serovar also possesses IS200 in the *flbD-flaA* intergenic region (Figure 3).

Primers Sense-59 and Antisense-83 amplify the *flbB* allele [26]. Primer Sense-59 binds at position 258 and primer Antisense-83 binds at position +100 of the 5’-3’ consensus *flbB*1,2 sequence. These primers amplify a 1389-bp product from strains that possess a Phase 2 flagellar antigen and no product from strains that lack a Phase 2 flagellar antigen such as I 4,[5],12:i:-. As shown in Figure 3, the PCR was able to discriminate between *Salmonella Typhimurium* and I 4,[5],12:i:- strains and other serogroup B, H:i serovars except *Salmonella Farsta*.

We tested all the *Salmonella Typhimurium*, I 4,[5],12:i:- and I 4,[5],12:NM strains identified in Mali and found that 170 of 171 *Salmonella Typhimurium* strains were correctly identified (i.e., possessed a 1-kb *flbB-flaA* intergenic region product and *flbB*1,2), and 51 of 52 I 4,[5],12:i:- strains were correctly identified (i.e., possessed a 1-kb *flbB-flaA* intergenic region product and lacked *flbB*1,2) (Table 4). The nine I 4,[5],12:NM strains produced mixed results in that all nine strains produced a 1-kb *flbB-flaA* intergenic region product but three strains possessed *flbB*1,2.

**Detection of *Salmonella Stanleyville***

Since *Salmonella Stanleyville* was found to be fairly common among the Mali NTS isolates, we added primers to detect *flcA-z4,23* of *Salmonella Stanleyville* to the multiplex PCR containing primers H-for, H-i, sdfF, sdfR, 16SF and DG74. The primers were first tested on *Salmonella Stanleyville* by themselves and produced a 427-bp amplicon. The *flcA-z4,23* primers were then added to the multiplex primer mix and PCR was performed (using the previously optimized conditions) on all 26 *Salmonella Stanleyville* strains, and a sample of 10 *Salmonella Typhimurium*, 10 *Salmonella Dublin* and 11 *Salmonella Enteritidis* strains. Correct amplicons were observed for all the strains tested. Figure 4 shows amplicons from a sample of three *Salmonella Stanleyville* strains and the control *Salmonella Typhimurium*, *Salmonella Enteritidis* and *Salmonella Dublin* strains.

**Discussion**

We have combined published primers and new primers in a multiplex PCR that, following the application of a previously described O serogrouping multiplex PCR [18], can identify *Salmonella Typhimurium* (and variants), *Salmonella Enteritidis*, *Salmonella Dublin* and *Salmonella Stanleyville*. Detection of *Salmonella Typhimurium*, *Salmonella Dublin* and *Salmonella Stanleyville* is based on amplification of the respective *flcA* alleles. We were unable to design primers to detect *flcA-g,m* or *Salmonella Enteritidis* due to the high nucleotide identity between *flcA-g,m* and *flcA-g,p* (of *Salmonella Dublin*). We therefore used primers to detect “Salmonella difference fragment I” (Sdf I), a segment of *Salmonella Enteritidis* DNA that was reported to be absent from 73 non-Enteritidis *Salmonella enterica* isolates comprising 34 different serovars as determined by PCR [27]. We confirmed the utility of Sdf I, with the exception of serovars Meleagridis and Livingstone. We found that *Salmonella Livingstone* yielded a weak PCR product using the same Sdf I primers that were previously reported [27]. The disparity could be due to a difference in the

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### Table 3. Detection of *Salmonella Typhimurium* and variants, *Salmonella Enteritidis* and *Salmonella Dublin* by multiplex PCR among 69 non-Typhi Group D *Salmonella* and 258 Group B *Salmonella* isolated from blood cultures of febrile patients in Bamako, Mali.

| O Group | Serovar | No. of isolates | Multiplex PCR |
|---------|---------|----------------|--------------|
|         |         |                | flcI | flcA-g,m | Sdf I |
| D       | Dublin  | 37             | 37   | 0       | 0    |
|         | Enteritidis | 32       | 0    | 0       | 32   |
| B       | Typhimurium and Typhimurium variants | 232 | 232 | 0       | 0    |
|         | Stanleyville | 26   | 0    | 0       | 0    |

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amplification method (different polymerases and cycling conditions were used).

From the epidemiologic and public health perspective, being able to detect strains that are genetically similar to _Salmonella Typhimurium_ yet that constitute distinct serovars (i.e., I 4,[5],12:i:- and I 4,[5],12:NM) is important (e.g., for outbreak investigations). In the USA and Europe such strains are increasingly being reported [28–30]. In Spain, I 4,[5],12:i:- was the fourth most commonly isolated _Salmonella_ serovar from humans from 1998–1999 [29] and several studies suggest that this monophasic serovar is a variant of _Salmonella Typhimurium_ [24,31–33]. The PCR that we have described can generally discriminate the diphasic _Salmonella Typhimurium_ serovar (I 4,[5],12:i:1,2) from monophasic (I 4,[5],12:i:-) variants. Only one _Salmonella Typhimurium_ was misidentified as I 4,[5],12:i:- and vice versa. It is possible that our PCR will not be able to detect some serologically monophasic I 4,[5],12:i- strains as lack of Phase 2 flagellar antigen expression can be due to a variety of mechanisms ranging from point mutations to partial or complete deletions in _fljB1,2_ and adjacent genes. Additionally, if there is a deletion in the first 250 bp of _fljB1,2_, the primers we have chosen will not identify the strain as I 4,[5],12:i-. Furthermore, our PCR scheme cannot differentiate between _Salmonella Typhimurium_ and _Salmonella Farsta_. However, in practical terms, this is unlikely to pose a problem as _Salmonella Farsta_ is extremely rare.

One small set of strains where our PCR gives differing results from traditional serological methods are _Salmonella Typhimurium_-like non-motile variants (I 4,[5],12:NM). Notably, all nine Malian strains identified by serotyping methods as I 4,[5],12:NM were found to possess the _fliC-i_ allele and three of the strains also possessed the _fljB1,2_ gene. Two quite distinct explanations can account for these observations. One is that in some strains lack of motility is not due to loss of flagellar genes but rather to other factors (e.g., regulation) that keep expression turned off. Alternatively, it may be that our genetic identification of these strains is correct and that the failure to detect flagella phenotypically is merely a consequence of not knowing how to grow the bacteria under conditions optimal for expression of those flagella. We assume that the I 4,[5],12:NM strains from Mali are _Salmonella_...
Typhimurium variants as they possess flIC-i and IS200 in the flIB-flIA intergenic region. It is also possible, albeit unlikely, that they could be the very rarely isolated *Salmonella* Farsta.

Soyer et al. [34] have reported that there are at least two common clones of I 4,[5],12:i:- with different genomic deletions (an ‘American’ deletion genotype and a ‘Spanish’ deletion genotype). Both I 4,[5],12:i:- clones completely lack flIB and flIA. Preliminary analysis of the deletion using a variety of primers that amplify different sections of the flIB1,2 gene indicates that the I 4,[5],12:i:- strains from Mali appear to possess the 3’ end of flIB

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**Table 5.** *Salmonella* serovars identified by the PCRs described in this report and in Levy et al. [18].

| O Group | Vi | Phase 1 H flagella | Sdf I | flIB-flIA intergenic region | d-tartrate fermentation | Serovar |
|---------|----|--------------------|-------|-----------------------------|------------------------|---------|
| Typhoidal serovars | | | | | | |
| A | – | a | – | 250 bp | + | ND* | Paratyphi A |
| B | – | b | – | 250 bp | + | – | Paratyphi B sensu stricto |
| D | + | d or j | – | 250 bp | – | ND | Typhi |
| Non-typhoidal serovars | | | | | | |
| B | – | i | – | 1 kb | + | ND | Typhimurium and Farsta |
| B | – | i | – | 1 kb | – | ND | I 4,[5],12:i:- |
| B | – | a4,a23 | – | 250 bp | – | ND | Stanleyville |
| D | +/- | g,p | – | 250 bp | – | ND | Dublin |
| D | – | + | 250 bp | – | ND | Enteritidis |

*ND, not determined.*

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**Figure 5.** The steps used to identify typhoidal and non-typhoidal serovars of *Salmonella* isolated from blood.

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and the entire fljA ORF. At least 250 bp of fljB (including the Sense-59 binding site) has been deleted at the 5’ end (data not shown). This suggests that these strains are genetically different from both the Spanish and American I 4,[5],12:i:- isolates. We are sequencing the deletion in several Malian I 4,[5],12:i:- strains to determine the exact deletion. It will be interesting to see whether I 4,[5],12:i:- strains from other African countries are genetically similar to the Malian strains.

Several other DNA-based Salmonella typing methods have been described [35–41]. However, some of these do not identify the breadth of enteric fever and NTS serovars of our multistep, multiplex PCR or fail to include an internal positive control. An O serogroup-specific Bio-Plex assay to detect serogroups B, C1, C2, D, E and O13 and serovar Paratyphi A [42] and a DNA sequence-based approach to serotyping have also been described [43]. However, these methods require greater financial and technical resources over those required for our method. Our PCRs are novel because they use as few primers as possible to identify the most common non-typhoidal Salmonella serovars isolated from blood and other invasive sites in sub-Saharan Africa, including Salmonella Typhimurium (and several variants), Salmonella Entertiditis, Salmonella Dublin and Salmonella Stanleyville. Since the late 1980s, the majority (85 to 95%) of NTS associated with invasive disease in sub-Saharan Africa belong to these serovars [9,11–13,15,16,18,19]. Therefore, we do not believe that there is a need for multiplex PCRs that detect more serovars unless the epidemiologic picture changes. We have tried to keep the PCRs as simple as possible so that they can be performed easily and the results interpreted correctly in laboratories in Africa that may be new to PCR. If a large outbreak or otherwise frequent isolation occurred of a serovar not presently recognized or contained within our multiplex, this serovar would not be identifiable using our PCR and would have to be identified in a reference laboratory using antisera or by molecular serotyping.

We are currently evaluating various PCR reagents that are stable at room-temperature and can be readily obtained by laboratories in Africa. Depending on the prevalence of certain serovars in a given country, either typhoidal or non-typhoidal Salmonella (or both) can be identified using our primer sets (Table 5 and Figure 5). For example, one may wish to test all Salmonella isolates in the O serogrouping PCR, then screen serogroup A, B and D Vi+ strains using the first H typing multiplex PCR to identify Salmonella Typhi, Salmonella Paratyphi A and Salmonella Paratyphi B. The d-tartrate fermentation PCR can be performed to differentiate Salmonella Paratyphi B sensu stricto strains from Salmonella Paratyphi B Java. Any serogroup B isolates not identified by the 1st H typing PCR can be tested along with non-Typhi O serogroup D strains in the second H typing/Sdf I multiplex PCR to identify serovars Typhimurium (and related strains), Dublin (which can be Vi+ or Vi- [44]), Enteritidis and Stanleyville. The O serogroup B H1 strains can be tested using the Typhimurium/I 4,[5],12:i:- PCR to identify Salmonella Typhimurium and I 4,[5],12:i:-. It should be stressed that the O serogrouping PCR described by Levy et al. [19] needs to be performed in conjunction with the PCRs described here to ensure that Salmonella Enteritidis and Salmonella Typhimurium are identified correctly and not mistaken as Salmonella Meleagrindis and Salmonella Livingstone; and Salmonella Cotham, respectively.

The surveillance experience in Mali is the first to show that Salmonella Dublin and Salmonella Stanleyville can constitute important serovars associated with invasive non-typhoidal Salmonella disease, along with Salmonella Typhimurium (and variants) and Salmonella Enteritidis. Previously, Salmonella Dublin and Salmonella Stanleyville were recovered only occasionally from blood cultures of patients in Africa [12,13,45,46]. We thought it useful to be able to detect these serovars by PCR in future surveillance studies in Africa.

In conclusion, we have described a series of PCRs based on O serogrouping and H typing that can identify the causative agents of enteric fever (Salmonella Typhi and Salmonella Paratyphi A and Salmonella Paratyphi B), the three most commonly isolated serovars that cause invasive disease in young children in sub-Saharan African (Salmonella Typhimurium [and Typhimurium-like], Salmonella Enteritidis and Salmonella Dublin) and Salmonella Stanleyville, an invasive pathogen that may be of regional importance in West Africa.

Supporting Information

Alternative Language Abstract S1  French translation of the abstract by SOS.

Found at: doi:10.1371/journal.pntd.0000621.am1 (0.02 MB DOC)

Author Contributions

Conceived and designed the experiments: SMT HL MML. Performed the experiments: SMT SD HL SL SOS MT PIF BT JPN MML. Contributed reagents/materials/analysis tools: SMT SD HL SL SOS MT MM BT KLK JEG. Analyzed the data: SMT HL SL SOS MT PIF MM KLK JPN. Wrote the paper: SMT MML.

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