JOURNAL OF CLINICAL MICROBIOLOGY, May 2008, p. 1861–1866 Vol. 46, No. 5
0095-1137/08/$08.00+0 doi:10.1128/JCM.00109-08


# PCR Method To Identify *Salmonella enterica* Serovars Typhi, Paratyphi A, and Paratyphi B among *Salmonella* Isolates from the Blood of Patients with Clinical Enteric Fever▽

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Received 18 January 2008/Returned for modification 3 March 2008/Accepted 11 March 2008

**PCR methodology was developed to identify *Salmonella enterica* serovars Typhi, Paratyphi A, and Paratyphi B. One multiplex PCR identifies serogroup D, A, and B and Vi-positive strains; another confirms flagellar antigen "d," "a," or "b." Blinded testing of 664 Malian and Chilean *Salmonella* blood isolates demonstrated 100% sensitivity and specificity.**

Identification of the serovars of *Salmonella* isolated from blood cultures, the lynchpin of enteric fever surveillance, is problematic in developing countries. Classical methods require high-quality O grouping and H typing antisera, reagents that can be difficult to obtain consistently. Accordingly, reference and research laboratories in developing countries, as in industrialized countries, are turning to multiplex PCR methods as a consistent, high-throughput approach to typing etiologic agents (1, 3, 25, 26). We utilized three sequential PCRs to identify the three classical pathogens that cause enteric fever, *Salmonella* serovars Typhi, Paratyphi A, and Paratyphi B, as an alternative to serotyping. An O grouping multiplex PCR identifies groups A, B, and D, based on described primers (12, 20). An H typing multiplex developed for this work identifies phase 1 H types "a," "b," and "d." A third PCR uses primers described below to identify serovar Paratyphi B biovar Java that ferments *d*-tartrate (dT) (22). The sequential PCR methodology is robust and amenable to high throughput for use in research and reference laboratories in developing countries.

Classical *Salmonella* serovar Typhi reference strains expressing H antigen (d) and unusual strains expressing antigen "j" are listed in Table 1, along with reference *Salmonella* serovar Paratyphi A and B strains and 24 "negative control" strains of other serovars.

Two sets of putative *Salmonella* strains isolated from blood cultures of febrile patients were tested. One set included 443 isolates obtained from blood cultures of 431 febrile patients at l'Hôpital Gabriel Touré in Bamako, Mali, in the course of systematic surveillance for bacteremia and invasive bacterial disease among patients younger than 16 years of age with fever, who were admitted to the hospital or seen in the emergency room (6, 27). Strains initially identified in the clinical microbiology laboratory of the Centre pour le Développement des Vaccins, Bamako, Mali (CVD-Mali), as *Salmonella* serotype Typhi, Paratyphi A, or Paratyphi B or as *Salmonella* species were shipped to CVD-Baltimore for bacteriological confirmation. The second set was 34 putative *Salmonella* serovar Paratyphi A and 189 *Salmonella* serovar Paratyphi B strains isolated from blood cultures of patients in the course of surveillance for enteric fever in Santiago, Chile (4, 8, 9, 15–18).

The serovars of the vast majority of Malian and Chilean isolates were identified at CVD-Baltimore by agglutination with O grouping (Denka Seiken Co. Ltd, Japan) and H typing antisera (Sifin Institute, Berlin, Germany) (5, 7); remaining isolates were serotyped by the CDC *Salmonella* Reference Laboratory. CVD-Baltimore and CDC clinical microbiology results were the "gold standard" against which the performance of the multiplex PCR methods was compared to assess their sensitivity, specificity, and positive predictive value.

The 5′ to 3′ sequences for the various primers and the sizes of the expected amplicons are presented in Table 2. To ensure that the absence of PCR products was not a failure in the PCR per se, an internal control (P1 and P2 that amplify *oriC*) (23) was incorporated into the system (13). The primers in the O serogrouping multiplex have previously been described (12, 20), but heretofore were used in two distinct protocols. We combined these primers into a single multiplex PCR by optimizing primer concentration, annealing temperature, and elongation time.

The H typing primers were designed by using sequences from GenBank (accession numbers AE014613, X03393, and AY649698) so that the sizes of the DNA fragments would enable facile recognition on 1 to 2% agarose gels. The primers dT-for and dT-rev detect the gene encoding the proficient active enzyme that allows dT fermentation by the Java biovar (22).

Three bacterial colonies were suspended in 100 μl of double-distilled water in 0.5-ml PCR tubes. Tubes were placed in

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▽ Published ahead of print on 26 March 2008.

TABLE 1. Twenty-two *Salmonella* serovar Typhi, Paratyphi A, and Paratyphi B reference strains and 24 "negative control" *Salmonella* strains consisting of other serovars used for preliminary validation of the multiplex PCR assays

| Strain | *Salmonella* serovar | O group | Phase 1 H flagellar antigen(s) | Vi capsular antigen status | dT fermentation status[a] | |
|---|---|---|---|---|---|---|
| | | | | | Jordan's | Kauffmann's |
| Ty2 | Typhi | D | d | + | NT | NT |
| CVD 908-*htrA* | Typhi | D | d | + | NT | NT |
| CDC 06-0418 | Typhi | D | j | + | NT | NT |
| CDC 01-0274 | Typhi | D | j | + | NT | NT |
| CDC 2433 | Typhi | D | j | + | NT | NT |
| CDC 96-0344 | Typhi | D | j | + | NT | NT |
| 01-0020 | Paratyphi A | A | a | − | NT | NT |
| 01-0220 | Paratyphi A | A | a | − | NT | NT |
| 02-0209 | Paratyphi A | A | a | − | NT | NT |
| 02-0532 | Paratyphi A | A | a | − | NT | NT |
| 04-0529 | Paratyphi A | A | a | − | NT | NT |
| 04-0571 | Paratyphi A | A | a | − | NT | NT |
| 05-0741 | Paratyphi A | A | a | − | NT | NT |
| 00-0391 | Paratyphi B | B | b | − | − | − |
| 02-0303 | Paratyphi B | B | b | − | − | − |
| 04-0137 | Paratyphi B | B | b | − | − | − |
| 04-0615 | Paratyphi B | B | b | − | − | − |
| 00-0301 | Paratyphi B | B | b | − | + | + |
| 01-0399 | Paratyphi B | B | b | − | + | + |
| 03-0451 | Paratyphi B | B | b | − | + | + |
| 04-0126 | Paratyphi B | B | b | − | + | + |
| 01-0516 | Paratyphi B | B | b | − | + | + |
| CDC 32 | Paratyphi C | C1 | c | − | NT | NT |
| CDC 33 | Paratyphi C | C1 | c | − | NT | NT |
| 06-0868 | Choleraesuis (sensu stricto) | C1 | c | − | NT | NT |
| 06-0894 | Choleraesuis subsp. Kunzendorf | C1 | c | − | NT | NT |
| 06-0707 | Dublin | D | g, p | − | NT | NT |
| State lab 81.23500 | Typhimurium | B | i | − | NT | NT |
| CDC 07-0100 | Braenderup | C1 | e, h | − | NT | NT |
| CDC 07-0222 | Tennessee | C1 | z29 | − | NT | NT |
| CDC 07-0230 | Tennessee | C1 | z29 | − | NT | NT |
| CDC 07-0108 | Orion | E1 | y | − | NT | NT |
| CDC 07-0044 | Newport | C2 | e, h | − | NT | NT |
| CDC 07-0042 | Montevideo | C1 | g, m, s | − | NT | NT |
| CDC 07-0039 | Bardo | C2 | e.h | − | NT | NT |
| CDC 07-0021 | Give | E1 | l, v | − | NT | NT |
| CDC 07-0115 | Meleagridis | E1 | e.h | − | NT | NT |
| CDC 07-0124 | Mbandaka | C1 | z10 | − | NT | NT |
| CDC 07-0217 | Oranienburg | C1 | m, t | − | NT | NT |
| CDC 07-0223 | Virginia | C2 | d | − | NT | NT |
| CDC 07-0001 | Cotham | O28 | i | − | NT | NT |
| CDC 07-0006 | Edinburg | C1 | b | − | NT | NT |
| CDC 07-00014 | Livingstone | C1 | d | − | NT | NT |
| CDC 07-0019 | Inverness | O38 | k | − | NT | NT |
| CDC 07-0094 | Choleraesuis subsp. Kunzendorf | C1 | c | − | NT | NT |
| CVD SE | Enteritidis | D | g, m | − | NT | NT |

[a] NT, not tested.

a PCR machine, incubated at 95°C for 10 min, and cooled to 25°C. The cell debris was pelleted by centrifugation at 16,000 × *g* for 30 s, and 5 μl of clear supernatant was used as the template in a PCR. This constituted "crude DNA." "Purified DNA" was prepared by hot phenol treatment (19). A dilution of 1:50 was made in double-distilled water, and 5 μl of the diluted DNA was used for the PCRs.

PCR was performed in 1× PCR buffer, 3.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates, and 0.2 U of Invitrogen *Taq* DNA polymerase (final volume of 25 μl) in a Mastercycler (Eppendorf North America, Westbury, NY). Primers were combined at a concentration of 5 μM each (final concentration of 0.2 μM), except for the positive control primers (*oriC*) that were used at a concentration of 3.5 μM (final concentration of 0.14 μM) in the H mix. For each PCR, 1.0 μl of mix was used per reaction.

The cycling parameters of the PCRs were as follows. The O grouping multiplex PCR consisted of denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final step of 72°C for 5 min. The H typing multiplex PCR comprised a denaturation step of 2 min at 95°C, followed by 35 cycles of the following two steps: 95°C for 30 s and 55°C for 15 s. The dT fermentation PCR consisted of a denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s and 60°C for 30 s. PCR products were separated on 2% (wt/vol) agarose gels, stained with ethidium bromide

TABLE 2. Primers used in the multiplex PCR assays and the expected amplicons

| Primer | Primer sequence (5′ to 3′) | Amplicon size (bp) | Reference |
|---|---|---|---|
| O serogrouping | | | |
| rfbJ-for | CCAGCACCAGTTCCAACTTGATAC | 662 | 20 |
| rfbJ-rev | GGCTTCCGGCTTTATTGGTAAGCA | | |
| tyv-for | GAGGAAGGGAAATGAAGCTTTT | 614 | 12 |
| tyv-rev | TAGCAAACTGTCTCCCACCATAC | | |
| vi-for | GTTATTCAGCATAAGGAG | 439 | 12 |
| vi-rev | CTTCCATACCACTTTCCG | | |
| prt-for | CTTGCTATGGAAGACATAACGAACC | 256 | 12 |
| prt-rev | CGTCTCCATCAAAAGCTCCATAGA | | |
| H antigen typing | | | |
| H-for | ACTCAGGCTTCCCGTAACGC | | This study |
| Ha-rev | GAGGCCAGCACCATCAAGTGC | 423 | This study |
| Hb-rev | GCTTCATACAGACCATCTTTAGTTG | 551 | This study |
| Hd-rev | GGCTAGTATTGTCCTTATCGG | 763 (d) or 502 (j)[b] | This study |
| dT fermentation | | | |
| dT-for | GTAAGGGTAATGGGTTCC | 289 | 22 |
| dT-rev | CACATTATTCGCTCAATGGAG | | |
| Internal control[a] | | | |
| P1 (*oriC*) | TTATTAGGATCGCGCCAGGC | 163 | 31 |
| P2 (*oriC*) | AAAGAATAACCGTTGTTCAC | | |

[a] Internal controls were included in both multiplex mixes and monoplex PCRs.
[b] Letters in parentheses indicate antigens.

and visualized on a UV transilluminator. Generally, for each PCR experiment, phenol-extracted DNA from *Salmonella* serovar Typhi, *Salmonella* serovar Paratyphi A, and *Salmonella* serovar Paratyphi B was used as a positive control. A negative control consisting of no DNA was always included.

To validate preliminarily the PCR for the identification of enteric fever pathogens, 22 reference strains were analyzed (Table 1). Initially, two classical *Salmonella* serovar Typhi, seven *Salmonella* serovar Paratyphi A, four *Salmonella* serovar Paratyphi B dT nonfermenters (i.e., $dT^-$), and five *Salmonella* serovar Paratyphi B $dT^+$ strains were tested and the amplicons in Fig. 1 were generated. With the O grouping multiplex, *Salmonella* serovar Typhi strains produced specific PCR products with the *prt* primers and with the *tyv* primers, as expected of group D *Salmonella* (Fig. 1A and C) and the primers for Vi antigen synthesis, compatible with being *Salmonella* serovar Typhi. The *Salmonella* serovar Paratyphi A strains produced a PCR band with the *prt*, but not the *tyv*, primers, indicating that the strains are part of group A; the *Salmonella* serovar Paratyphi B strains yielded a PCR product only with the *rfbJ* primers, indicative of group B.

When these 18 *Salmonella* strains were tested with the H antigen multiplex PCR, the two *Salmonella* serovar Typhi strains yielded a PCR product indicative of flagellar antigen d, the seven *Salmonella* serovar Paratyphi A isolates yielded a PCR product designating flagellar antigen a, and the nine *Salmonella* serovar Paratyphi B isolates exhibited a product denoting flagellar antigen b (Fig. 1B and C). Only the *Salmonella* serovar Paratyphi B strains capable of fermenting dT yielded a positive PCR product in the PCR for dT fermentation (Fig. 2). These initial PCRs demonstrated that results were identical regardless of whether highly purified or crude DNA was used as the template, so crude template DNA was employed for all subsequent PCRs.

To preliminarily assess the specificity of the multiplex PCRs, we tested 24 negative control *Salmonella* strains consisting of serovars other than Typhi, Paratyphi A, or Paratyphi B (Table 1). The multiplex PCR correctly identified *Salmonella* serotypes Typhimurium as group B, Dublin and Enteritidis as group D, Virginia and Livingstone as flagellar antigen d positive, and Edinburg as flagellar antigen b positive. Since none of these serotypes were positive in both the O group and the H antigen multiplex PCRs, they were clearly not enteric fever serovars.

Four reference *Salmonella* serovar Typhi strains that express j flagellar antigen instead of d antigen were also tested (Table 1); the *fliC*-j allele results from a 261-bp deletion in *fliC*-d (10). The H-for and Hd-rev primers bind DNA external to this deletion to amplify a 502-bp fragment rather than the 763-bp d amplicon (Fig. 3). The presence or absence of antigen z66, encoded by $fljB^{z66}$ on a linear plasmid, has no effect on the amplification of *fliC*-j (2). The four *Salmonella* serovar Typhi j strains yielded O multiplex amplicons indistinguishable from those of *Salmonella* serovar Typhi strains that possess *fliC*-d.

Systematic blood culture surveillance in Bamako, Mali, yielded 443 *Salmonella* strains from 431 patients initially identified in a Malian clinical microbiology laboratory as *Salmonella* serovar Typhi, serovar Paratyphi A, or serovar Paratyphi B or as *Salmonella* species. The purification of the subcultures in Baltimore revealed that 12 Malian patients harbored two distinct *Salmonella* serovars in their blood. Classical serotyping revealed that of the 419 Malian patients whose blood cultures yielded a single isolate: 164 were group D *Salmonella* (101 serovar Typhi, 33 serovar Dublin, and 30 serovar Enteritidis); 246 were group B (210 serovar Typhimurium, 25 serovar Stanleyville, 4 serovar I 4,5,12:i:−, and 7 serovar I 4,5,12:nonmotile strains); six were group C1 (four serovar Paratyphi C, one serovar Choleraesuis subsp. Kunzendorf, and one serovar Vir-

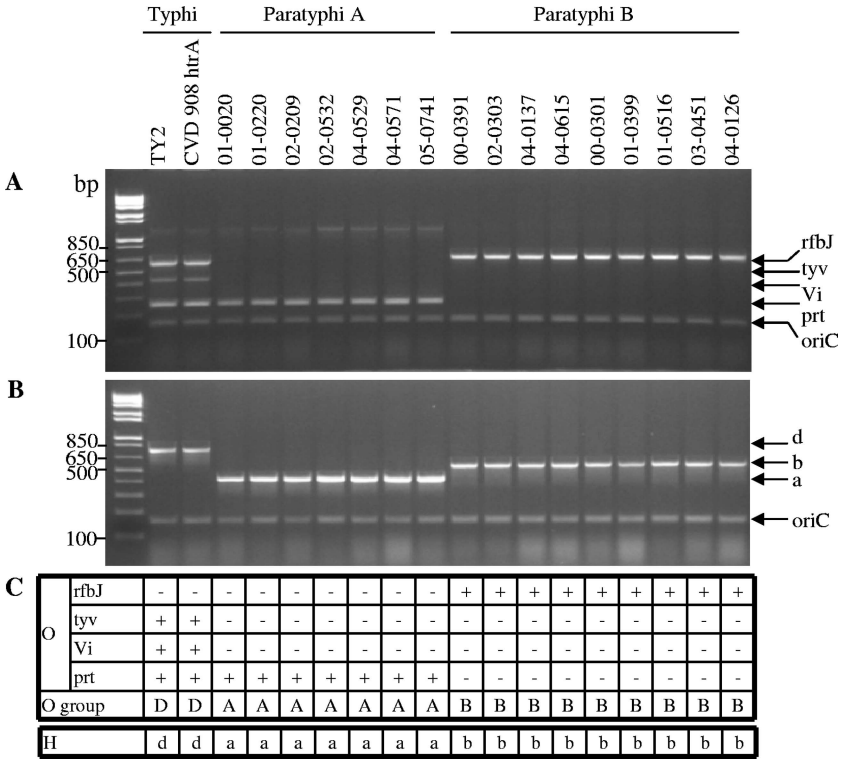


FIG. 1. Validation of the O grouping and H typing multiplex PCRs using 18 reference enteric fever strains. The sample consisted of two *Salmonella* serovar Typhi, seven *Salmonella* serovar Paratyphi A, and nine *Salmonella* serovar Paratyphi B strains. Crude DNA extracts were tested with the O multiplex PCR (A) and H multiplex PCR (B). (C) The interpretation of the PCR products.

chow); and the remaining three strains were serovars Havana (group O13), Minnesota (group O21), and Poona (group O13). Of the 12 patients whose blood cultures yielded two isolates, 6 harbored serovars Typhi and Typhimurium, 2 had serovars Typhimurium and Dublin, 2 had serovars Typhimurium and Enteritidis, 1 had serovars Dublin and I 4,5,12:nonmotile, and the last had serovars Stanleyville and Dublin. The 443 total strains from these 431 patients are summarized in Table 3.

The 443 Malian strains were coded before being tested blind with the multiplex PCRs to assess the sensitivity and specificity of these molecular diagnostic methods in identifying *Salmonella* serovar Typhi, serovar Paratyphi A, or serovar Paratyphi B (Table 3). All 107 *Salmonella* serovar Typhi strains were

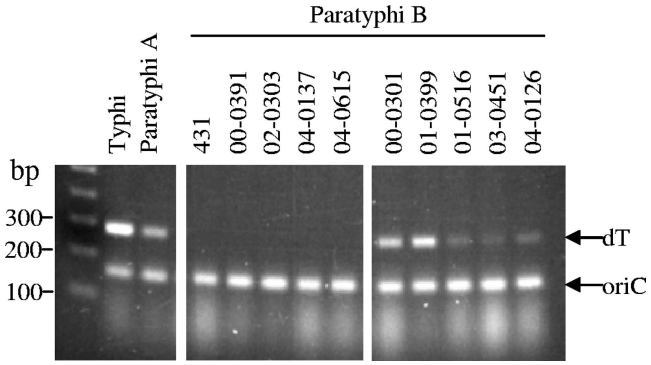


FIG. 2. Validation of the dT fermentation PCR using nine *Salmonella* serovar Paratyphi B reference strains. The PCR control strains were *Salmonella* serovar Typhi CVD 908-*htrA*, *Salmonella* serovar Paratyphi A ATCC 9150, and *Salmonella* serovar Paratyphi B 431. The *Salmonella* serovar Paratyphi B reference strains included five dT fermenters and four dT nonfermenters.

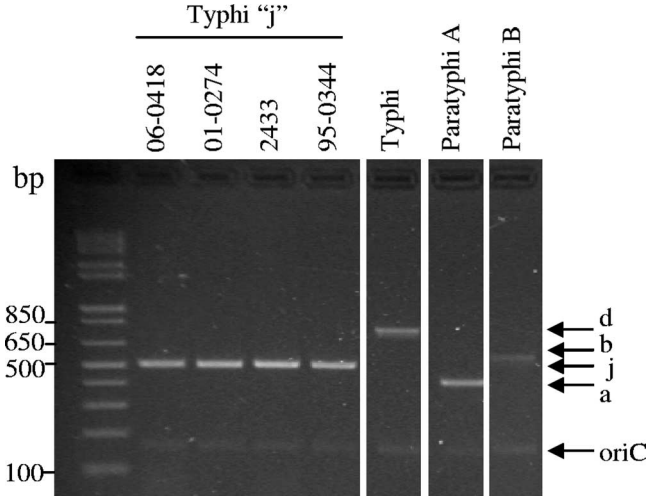


FIG. 3. Detection of rare *Salmonella* serovar Typhi strains that possess *fliC*-j by the H multiplex PCR. *Salmonella* serovar Typhi strains 06-0418, 01-0274, and 2433 possess *fliC*-j and *fljB*$^{z66}$. *Salmonella* serovar Typhi 95-0344 possesses *fliC*-j only. PCR control strains were *Salmonella* serovar Typhi CVD 908-*htrA*, *Salmonella* serovar Paratyphi A ATCC 9150, and *Salmonella* serovar Paratyphi B 431.

TABLE 3. Serovars of 443 *Salmonella* isolates from blood cultures of 431 febrile patients in Bamako, Mali, and results when tested in blinded fashion with the multiplex PCR assays

| *Salmonella* serovar | No. of isolates | No. of results by group in by indicated test | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | O group PCR | | | | Phase 1 H flagellar antigen PCR | | |
| | | D | Vi | B | A | d | b | a |
| Typhi | 107 | 107 | 107 | 0 | 0 | 107 | 0 | 0 |
| Dublin | 37 | 37 | 0 | 0 | 0 | 0 | 0 | 0 |
| Enteritidis | 32 | 32 | 0 | 0 | 0 | 0 | 0 | 0 |
| Serogroup B | | | | | | | | |
| Typhimurium | 220 | 0 | 0 | 220 | 0 | 0 | 0 | 0 |
| I 4,5,12:i:− | 4 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| I 4,5,12:nonmotile | 8 | 0 | 0 | 8 | 0 | 0 | 0 | 0 |
| Stanleyville | 26 | 0 | 0 | 26 | 0 | 0 | 0 | 0 |
| Serogroup C1 | | | | | | | | |
| Choleraesuis subsp. Kunzendorf | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Paratyphi C | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Virchow | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other serogroups | | | | | | | | |
| Havana | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Minnesota | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Poona | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

correctly identified as O group D, Vi positive, and positive for flagellar antigen d (100% sensitivity). The multiplex also detected all 69 of the non-serovar Typhi group D strains (37 serovar Dublin and 32 serovar Enteritidis). The O serogrouping multiplex PCR correctly detected all 258 of the group B strains (220 serovar Typhimurium, 12 serovar I 4,5,12:nonmotile, and 26 serovar Stanleyville strains). Although the precise serovar of these strains could not be determined in the molecular assay, they were clearly not enteric fever organisms. None of the remaining O serogroup strains (none of which were group A, B, or D) were positive in the O serogrouping PCR. The H typing multiplex correctly detected the b allele of the serovar Minnesota strain.

Among the Chilean isolates, 221 were *Salmonella*, including 26 isolates of *Salmonella* serovar Paratyphi A, 166 *Salmonella* serovar Paratyphi B, and 23 *Salmonella* serovar Typhi. The other six strains were serovars Anatum, Newport, Panama, Reading, Senftenberg, and Typhimurium (Table 4). The multiplex PCRs correctly identified all 26 *Salmonella* serovar Paratyphi A, 166 presumptive *Salmonella* serovar Paratyphi B, and 23 *Salmonella* serovar Typhi strains (Table 4). The O serogrouping PCR also detected the two group B serovars (Read-

TABLE 4. Serovars of 221 *Salmonella* isolates from blood cultures of 221 febrile patients in Santiago, Chile, and results when tested in blinded fashion with the multiplex PCR assays

| *Salmonella* serovar | No. of isolates | O group PCR | | | | Phase 1 H antigen PCR | | | Result of PCR to detect functional dT fermentation enzyme[a] |
|---|---|---|---|---|---|---|---|---|---|
| | | D | Vi | B | A | d | b | a | |
| Serogroup D | | | | | | | | | |
| Typhi | 23 | 23 | 23 | 0 | 0 | 23 | 0 | 0 | NT |
| Panama | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | NT |
| Serogroup A | | | | | | | | | |
| Paratyphi A | 26 | 0 | 0 | 0 | 26 | 0 | 0 | 26 | NT |
| Serogroup B | | | | | | | | | |
| Paratyphi B | 166 | 0 | 0 | 166 | 0 | 0 | 166 | 0 | 0 |
| Typhimurium | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | NT |
| Reading | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | NT |
| Other serogroups | | | | | | | | | |
| Anatum (E1) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | NT |
| Newport (C2) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | NT |
| Senftenberg (E4) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | NT |

[a] NT, not tested.

ing and Typhimurium) and the group D serovar (Panama), whereas the results for other serogroups (C2, E1, and E4) were all negative. All the *Salmonella* serovar Paratyphi B strains were of the dT-negative biotype.

Among the 664 clinical isolates tested from Mali ($n = 443$) and Chile ($n = 221$), there were 130 serovar Typhi, 26 serovar Paratyphi A, and 166 serovar Paratyphi B sensu stricto isolates. The multiplex PCRs proved 100% sensitive in detecting all of these true strains. The O serogrouping PCR was also 100% sensitive in detecting other serovars within groups B and D.

The specificity of the multiplex PCRs for enteric fever bacilli was also 100%, as none of the remaining 342 *Salmonella* strains belonging to 17 serovars were identified as an enteric fever serovar. The positive predictive value of the multiplex PCR method for detecting enteric fever *Salmonella* was 100%.

Since our first-step multiplex assay includes both O group and Vi antigen detection, *Salmonella* serovar Typhi can be presumptively identified at this stage. Although other organisms express Vi antigen, only *Salmonella* serovar Typhi and a subset of serotype Dublin represent Vi-positive group D strains. Strains positive for one of the three targets in the O grouping multiplex were then tested in the H multiplex. With the exception of some Indonesian strains that express H antigen j, all *Salmonella* serovar Typhi strains express antigen d (2, 10, 11, 14, 24, 28–30). Other Indonesian strains express "z66" antigen in addition to d or j.

A previously described multiplex PCR *Salmonella* typing method is not useful for the surveillance of enteric fever (21), since serotypes Paratyphi A and Paratyphi B would not be identified and, without further testing, serotype Paratyphi C cannot be differentiated from serotype Typhi. The fact that highly purified template DNA is not required for our three-tiered multiplex PCR typing system enhances the likelihood of successfully deploying this streamlined technology to laboratories in developing countries in Africa and Asia.

This research was supported by grant R01 AI029471 (to M. M. Levine) from the NIAID, NIH, and two grants from the Bill and Melinda Gates Foundation (to M. M. Levine).

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