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## Clinical Evaluation of a Multiplex PCR for the Detection of *Salmonella enterica* Serovars Typhi and Paratyphi A from Blood Specimens in a High-Endemic Setting

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1 **Clinical evaluation of a multiplex PCR for the detection of *Salmonella enterica* serovars**  
2 **Typhi and Paratyphi A from blood specimens in high endemic setting.**

3 \*Stephane Pouzol<sup>1</sup>, Arif Mohammad Tanmoy<sup>2,3</sup>, Dilruba Ahmed<sup>4</sup>, Farhana Khanam<sup>4</sup>, W. Abdullah  
4 Brooks<sup>5</sup>, Golam Sarower Bhuyan<sup>6</sup>, Laetitia Fabre<sup>7</sup>, Juliet E Bryant<sup>1</sup>, Marie-Paule Gustin<sup>1,8</sup>,  
5 Philippe Vanhems<sup>1,9</sup>, Bill Carman<sup>10</sup>, François-Xavier Weill<sup>7</sup>, Firdausi Qadri<sup>4</sup>, Samir Saha<sup>2</sup> and  
6 Hubert Endtz<sup>1,3</sup>

7  
8 <sup>1</sup>Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre International de Recherche  
9 en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de Lyon, UCBL1, Lyon, France.

10 <sup>2</sup>Child Health Research Foundation (CHRF), Dhaka, Bangladesh.

11 <sup>3</sup>Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The  
12 Netherlands.

13 <sup>4</sup>International Centre for Diarrhoeal Disease Research (icddr,b) Dhaka, Bangladesh.

14 <sup>5</sup>Bloomberg School of Public Health, Department of International Health, Johns Hopkins  
15 University, Baltimore, MD, USA.

16 <sup>6</sup>Institute for Developing Science and Health Initiatives (ideSHi,), Dhaka, Bangladesh.

17 <sup>7</sup>Institut Pasteur, Unité des Bactéries Pathogènes Entériques, Paris, France.

18 <sup>8</sup>Institute of Pharmacy, Department of Public Health, University of Lyon 1, Lyon, France

19 <sup>9</sup>Service d'Hygiène, Epidémiologie et Prévention, Hôpital Edouard Herriot, Hospices Civils de  
20 Lyon, Lyon, France.

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\*Stephane Pouzol. Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de Lyon, UCBL1, Lyon, France. Stephane.pouzol@fondation-merieux.org. +33 4 81 07 99 64

21 <sup>10</sup>Fast Track Diagnostics, Luxembourg.

22

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30 **ABSTRACT**

31 Enteric fever is a major public health concern in endemic areas, particularly in infrastructure  
32 limited countries where *Salmonella* Paratyphi A has emerged in increasing proportion of cases.  
33 We aim to evaluate a method to detect *S. Typhi* and *S. Paratyphi A* in febrile patients in  
34 Bangladesh. We conducted a prospective study enrolling patients with fever > 38°C admitted to  
35 two large urban hospitals and two outpatient clinics located in Dhaka, Bangladesh. We  
36 developed and evaluated a method combining short culture with new molecular assay to  
37 simultaneously detect and differentiate *S. Typhi* and *S. Paratyphi A* from other *Salmonella*  
38 directly from 2-4 mL of whole blood in febrile patients (n=680). A total of 680 cases were  
39 enrolled from the four participating sites. Increase detection rate (+ 38.8%) in *S. Typhi* and *S.*  
40 *Paratyphi A* was observed with multiplex PCR assay and absence of non-typhoidal *Salmonella*  
41 detection was reported. All 45 healthy controls were culture and PCR negative, generating an  
42 estimated 92.9% of specificity on clinical samples. When clinical performance was assessed in  
43 absence of blood volume prioritization for testing, a latent class model estimates clinical  
44 performance  $\geq 95\%$  in sensitivity and specificity with LR+ > 10 and LR- < 0.1 for the multiplex  
45 PCR assay. The alternative method to blood culture we developed may be useful alone or in  
46 combination with culture or serological tests for epidemiological studies in high disease burden  
47 settings and should be considered as secondary endpoint test for future vaccine trials.

48

## 49 INTRODUCTION

50 Enteric fever is a severe systemic infectious disease caused by human-restricted pathogens  
51 *Salmonella enterica* serovars Typhi (*S. Typhi*) or Paratyphi (*S. Paratyphi*) A, B or C.  
52 Transmission of the disease is both waterborne and foodborne, or through direct person to  
53 person contact. Use of antibiotics and improvements in sanitation and hygiene have almost  
54 eradicated enteric fever in high-income countries. However, the disease is still of utmost  
55 importance in Asia (> 90% cases) and Africa where nearly 21 million cases and more than  
56 220,000 deaths are estimated to occur annually <sup>1</sup>. In south Asia, the incidence of enteric fever  
57 was 394.2 episodes per 100,000 person-years in 2010 <sup>2</sup>, compared to 240 episodes per 100,00  
58 person-years of enteric fever reported in 2003 in a urban slum of Dhaka, Bangladesh <sup>3</sup>. Although  
59 enteric fever is frequently considered a disease of school children and young adults, population-  
60 based studies in Bangladesh have reported the highest incidence rate in children under 5 years  
61 (1870 episodes/100,000 person-years vs 210 episodes/100,000 person-years in older age  
62 groups), with higher prevalence of typhoid than paratyphoid <sup>3,4</sup>.

63 Due to self-medication with antimicrobials prior to consultations, the classic 'textbook'  
64 presentation of enteric fever with a slow 'step-ladder' rise in fever and toxicity is now rarely seen  
65 <sup>5</sup>. Consequently, differentiating enteric fever in endemic settings from others undifferentiated  
66 febrile illnesses such as influenza, leptospirosis, dengue or malaria, is very challenging,  
67 particularly in children who may present with atypical signs <sup>6</sup>. The World Health Organization  
68 recommends bacterial isolation from blood or bone marrow for definitive diagnosis of enteric  
69 fever <sup>7</sup>. However, because of its invasive nature, bone marrow aspirates are rarely collected  
70 although their culture yields good sensitivity ( $\approx$  90%) and is relatively refractory to prior  
71 antibiotics consumption of patients <sup>8</sup>. In current clinical practice in most endemic countries, blood  
72 culture and Widal test are the most common diagnostic procedures employed despite poor  
73 sensitivity and specificity <sup>9</sup>. Indeed, blood culture sensitivity has been estimated at 59% of

74 presumptive cases, ranging from 51% to 65% according to specimen volume <sup>10</sup>, while Widal test  
75 is hampered by lack of standardization of reagents and/or misuse and misinterpretation of  
76 results <sup>11,12</sup>. Serological rapid diagnostic tests like Typhidot-M or Tubex may represent some  
77 improvement over the Widal test, but still have suboptimal sensitivity and specificity <sup>13</sup>. Another  
78 diagnostic assay that shows promise is the TPTest <sup>14</sup>, that detects *S. Typhi* and *S. Paratyphi A*  
79 antibody secreted by isolated lymphocytes.

80 Polymerase Chain Reaction (PCR) methods have become central to infectious disease  
81 diagnostics, as they provide rapid, sensitive, and specific results that are unaffected by patient  
82 consumption of antibiotics. In enteric fever diagnosis, molecular tests have been initially  
83 developed targeting genes encoding somatic (O), flagellar (H) and Vi antigens of *S. Typhi* <sup>15-17</sup>,  
84 as well as *Salmonella* Pathogenicity Island 1 (*hilA*) <sup>18</sup>, 16sRNA gene <sup>19</sup>, or complexes thought to  
85 be important for the entry of salmonellae into enterocytes <sup>20</sup>. *In Silico* comparative genomics and  
86 advances in technologies have led to the improvement <sup>21-26</sup> or development of additional PCR  
87 assays <sup>27-31</sup>. Among *S. Typhi* culture-confirmed positives, PCRs sensitivity have been generally  
88 reported > 90% and specificity near 100% with a limit of detection determined as low as 4  
89 CFU/mL. However, microbiological data suggests that blood culture can be positive in patients  
90 with a median bacteremia count of 1 CFU/mL of blood <sup>32</sup>. *S. Paratyphi A* has emerged in an  
91 increasing proportion of enteric fever cases in some settings; the high variability in reported  
92 burden estimates in Asia suggests considerable geospatial variability in the burden of  
93 paratyphoid fever <sup>33</sup>. Studies conducted in Dhaka in 2003 indicated 16.7% cases of enteric fever  
94 were related to *S. Paratyphi A* <sup>3</sup>. Multiplexed PCR assays to simultaneously detect *S. Typhi* and  
95 *S. Paratyphi A* are therefore needed in Asia, however, the clinical performance of multiplexed  
96 PCR assays are frequently compromised relative to monoplex assays in sensitivity (40%) <sup>28,31</sup> or  
97 specificity (63%) <sup>27</sup>.

98 Because PCR performance is highly dependent on the amount of material to be amplified,  
99 combining a brief pre-enrichment blood culture followed by molecular detection is a promising  
100 strategy to increase sensitivity of detection <sup>34</sup>. In the current study, we developed a rapid assay  
101 combining bacterial pre-enrichment by blood culture and multiplex real-time PCR for detection of  
102 *Salmonella* serotypes Typhi and Paratyphi A. We describe clinical performance of the assay  
103 when used to diagnose suspected typhoid patients in Bangladesh.

## 104 **MATERIALS AND METHODS**

### 105 Design of the molecular assay

106 Based on a conventional PCR assay targeting CRISPR regions <sup>35</sup>, Fast-Track Diagnostics  
107 (FTD) and Institut Pasteur optimized the length of the amplified regions of *S. Typhi* and *S.*  
108 *Paratyphi A* to suit a multiplex real time PCR assay format. Taqman® probes were designed  
109 outside the direct repeat region of the CRISPR loci. These were FAM (*S. Typhi*) and YAK (*S.*  
110 *Paratyphi A*) labelled. *SopD* and *ttrAC* genes were used to define 2 additional sets of  
111 primers/probes (ATTO label) for *Salmonella enterica* (*S. spp*) identification. FTD manufactured  
112 the assay under Good Manufacturing Practices and included a positive control (plasmids  
113 containing the PCR targeted regions) and an Internal Control (inactivated *Streptococcus equi*)  
114 for qualification of extraction procedure (ROX label). FTD company commercialized the assay  
115 under the FTLYO-35-64-L reference.

### 116 Real-time PCR Method

117 The method developed combines a short incubation step to “enrich” the samples with a  
118 molecular multiplex PCR for pathogens identification. Briefly, an equivalent volume of TSB-5%  
119 bile (Becton Dickinson and Company, USA) was added to the blood sample prior 5h incubation  
120 (37°C with shaking). The mixture was then filled to 10mL with PBS 1x, and 3 µl of internal  
121 control (FTD, Luxembourg) was added prior DNA extraction with QIAamp DNA blood maxi kit

122 (Qiagen, Germany) according to the manufacturer's instructions except that elution was  
123 performed with 1 mL of buffer and re-eluted with the first eluate. 10 µL of nucleic acids were  
124 subjected to a real-time nucleic amplification using the FTD Enteric Fever Kit (FTD,  
125 Luxembourg) and the AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, USA).  
126 PCR reactions were carried out on CFX96 PCR machine (BioRad, USA) using the following  
127 conditions: 50°C 15 min, 95°C 10 min followed by 95°C 8 sec, 60°C 34 sec for 40 cycles.  
128 Positive and negative controls provided in the FTD Enteric Fever Kit were performed in the same  
129 run. PCR signals were analyzed using CFX Manager Software version 3.1 (BioRad, USA) and  
130 expressed as cycles threshold (*Ct*) values. To validate a result, a serovar specific signal (either  
131 *S. Typhi* or *S. Paratyphi A*) must be associated with detection of enterica specie (*S. spp*) except  
132 for NTS isolates whom are only positive for *S. spp* signal. The comparative PCR test, hereafter  
133 designated "Nga-PCR" assay, has been already described by Nga et al <sup>28</sup>. Briefly, the assay  
134 targets STY0201 and SSPA2308 genes and was slightly modified as follow: 25 µl reactions  
135 volume containing 15µl of Takyon kit for Probe qPCR Assays (Eurogentec, Liège, Belgium) with  
136 same primer (0.4µM) and probe (0.15µM) sets concentration and 10 µl of template DNA. PCR  
137 reactions were carried out on CFX96 PCR machine (BioRad) using the following conditions:  
138 95°C 3 min followed by 95°C 10 sec, 60°C 30 sec and 72°C 30 sec for 45 cycles.

### 139 *In vitro* performance of the molecular assay

140 For sensitivity experiments, *S. Paratyphi A* 1K and *S. Typhimurium* LT2 strains from *Institut*  
141 *Pasteur* and *American Type Culture Collection* (ATCC, USA) were respectively used.  
142 *Salmonella* isolates were grown overnight at 37°C in trypticase soy broth and adjusted to 0.1  
143 OD<sub>600</sub> Unit. After centrifugation (13000 rpm, 1 min), bacteria were suspended in 10-fold serial  
144 dilutions in PBS and suitable dilutions were then used to seed either whole blood (*Etablissement*  
145 *français du sang*, France) w/wo TSB-ox bile 5% (Beckton Dickinson, USA) or PBS. Specificity of  
146 detection was evaluated using diverse isolates selected to be representative of global



147 *Salmonella* variation as previously described<sup>35</sup> : 90 *Salmonella* Typhi isolates, 33 *Salmonella*  
148 Paratyphi A isolates, and 16 Non Typhoid *Salmonella* (NTS) isolates. In addition, 39 different  
149 non-*Salmonella* bacterial isolates were tested, representing species frequently involved in non-  
150 typhoid enteric disease.

#### 151 Study sites and participants

152 We selected 4 study sites in Dhaka, Bangladesh including 2 hospitals (Dhaka Shishu Hospital,  
153 DSH and International Centre for Diarrhoeal Disease Research, Bangladesh, icddr,b) and 2 out-  
154 patient clinics located in urban slums of Dhaka (Mirpur and Kamalapur). From August 2014 to  
155 June 2015, adults and children > 2 years old with documented fever > 38°C for 3 consecutive  
156 days were recruited for the study. Exclusion criteria were fever with clinical signs indicating a  
157 clear focus of infection; inability to collect the required volume of blood; and refusal to consent.  
158 Controls were identified from hospital surgery wards or outpatient wards, with no history of fever  
159 in post- and prior 7 days of blood sampling and informed consent.

160 DSH and icddr,b laboratories performed blood culture, microbiology analysis and pre-enrichment  
161 and both conduct external quality assurance program (EQA) from UK National External Quality  
162 Assessment Service (UK NEQAS) and College of American Pathologists (CAP) respectively.  
163 Iccdr,b is ISO 15190 certified since 2011 and DSH is the reference lab in the SEARO region for  
164 WHO.

#### 165 Blood Collection and culture.

166 The recommended blood withdrawal volume for testing was stratified in three different groups  
167 based on age (2 to 5, 5 to 17 and > 17 years old). 3 mL of blood was collected to perform PCR  
168 in all age groups while 3 mL (2 to 4 years old group) to 5 mL was withdrawn for culture. An  
169 additional volume (1 mL) was collected to carry out a secondary end-point test (TP Test, data

170 not included). Moreover, some of the adult patients consented to participate in an additional  
171 study to evaluate an alternative test, and for these individuals an additional volume of blood (5  
172 mL) was collected. Blood Culture was performed with either BACTEC (Becton Dickinson and  
173 Company, USA) or BacT/Alert (bioMérieux, France) automated culture system at DSH and  
174 icddr,b respectively. Each bottle of standard aerobic medium was filled with blood specimen and  
175 incubated until growth was detected. We evaluated the volume of blood by measuring the weight  
176 of the bottle before and after inoculation. A visual inspection was performed at the end of the  
177 incubation period (5 days as recommended by the manufacturer) if growth had not already been  
178 automatically detected by the instrument. Any positive samples were subcultured on sheep  
179 blood agar and MacConkey agar plates, and putative *S. Typhi*/*Paratyphi-A* colonies were  
180 identified by biochemical test and agglutination with Remel Agglutinating Sera (Thermo Fisher  
181 Scientific, USA).

#### 182 Clinical Study Laboratory Method

183 In parallel to BC, an additional blood specimen was collected for each participant and  
184 immediately mixed with an equivalent volume of TSB-5% bile at lab arrival (weight based). The 5  
185 hours pre-enrichment step was carried out without delay prior -80°C storage and shipment. DNA  
186 extraction and multiplex PCR amplification were performed at Fondation Mérieux laboratory  
187 following the protocol previously described.

#### 188 Ethics

189 The study was approved by ethical committees of the Bangladesh Institute of Child Health  
190 (BICH-ERC-2/6/2012) and icddr,b (PR#13014-27/02/2013).

#### 191 Statistical analysis

192 Categorical variables were described as number, continuous variables as median and  
193 interquartile range (IQR); they were compared respectively by Mann-Witney U-test or Kruskal–  
194 Wallis in one-way analysis of variance, as appropriate. R (<http://www.R-project.org>) version 3.4.4  
195 software were used for data computation and analysis. R software with epiR package was used  
196 to calculate accuracy and odds ratio of the PCRs with blood culture as gold standard. Bayesian  
197 Latent Class Model (LCM) was performed with MPlus software version 7.11.

## 198 **RESULTS**

### 199 *In vitro* performance of the multiplex real-time PCR assay

200 Using serially diluted *Salmonella* strains spiked in either PBS or whole blood, we assessed the *in*  
201 *vitro* sensitivity of the multiplex real-time PCR assay (Table 1). All the standard curves  
202 demonstrated good analytical performance with  $R^2 > 0.980$  and PCR efficiency in the range of  
203 90-110%. No significant variation in the *Ct* value was observed on DNA extracted from whole  
204 blood or PBS ( $p > 0.2$  with non-parametric t-test) reflecting no influence of potential blood  
205 inhibitors on the PCR assay. The latest dilution with 100% of detected repetitions has been  
206 considered to define a reliable limit of detection (LOD). Although able to detect 1 CFU/reaction,  
207 the assay demonstrated consistent detection of replicates as low as 10 CFU/reaction for spiked  
208 bacteria in PBS and whole blood.

209 We also determined whether the molecular assay could detect other *Salmonella* serovars or any  
210 non-*Salmonella* bacteria, using clinical isolates and strains frequently identified in blood culture  
211 or involved in non-typhoid enteric disease (Supplementary Table 1). Although PCR results  
212 demonstrated cross-detection for a few isolates (1 *S. Paratyphi* A and 2 NTS with *Ct* value  $> 32$ ),  
213 the *Ct* value for the specific target was low (range 14.4 - 19.8), indicating a very high bacterial  
214 DNA concentration not in line with what could be expected on a biological sample.

215 Consequently, when diluted 10x or 100x, the molecular assay detected 100% of the *Salmonella*  
216 Typhi, Paratyphi A and NTS isolates and no cross detection with other enterobacteria.

#### 217 Evaluation of the multiplex real-time PCR assay in a Typhoid-endemic population

218 A total of 680 patients with suspected enteric fever and 45 healthy controls were enrolled from 4  
219 sites in Dhaka, Bangladesh between 2014 and 2015, including 80.7% (n=549)  $\leq$  17 years of age  
220 (Table 2). All 45 healthy controls remained negative with both PCR and culture. Results based  
221 on BC indicated a 14% (98/680) prevalence of the disease. PCR positivity was significantly  
222 correlated with shorter fever duration at time of presentation ( $p < 0.01$ ) but not to age, nor to  
223 differences in sample volume used for culture or molecular detection. Briefly, 136/680 suspected  
224 cases (20%) were positive by PCR (118 *S. Typhi* & 18 *S. Paratyphi A*) with no discrepancy in  
225 identification, whereas 98 (14%) were blood culture-positive (79 *S. Typhi* & 19 *S. Paratyphi A*).  
226 Seven culture-positive samples (4 *S. Typhi* & 3 *S. Paratyphi A*) were not identified with PCR  
227 (1%) and exhibited a longer time to positivity in culture ( $p < 0.005$ ). The assay showed capacity  
228 to detect additional (43 *S. Typhi* and 2 *S. Paratyphi A*) samples in negative BC samples.

229 Prior to evaluating performance in the clinical setting, we benchmarked the multiplex PCR assay  
230 with the only similar test available in 2014 <sup>28</sup> (Table 3), hereafter designated the “Nga-PCR”  
231 assay. Nga-PCR detected an additional (n=49) cases in negative BC samples but, similarly to  
232 the multiplex PCR assay, Nga-PCR also failed to detect the same 7 BC positive samples that  
233 required longer incubation times, mentioned above. Nga-PCR assay identified 81/98 multiple  
234 PCR-confirmed cases, especially notable for *S. Typhi* (65/79 vs 75/79), but failed in typing 4  
235 samples because of a specific signal identification for both *S. Typhi* and *S. Paratyphi A*.  
236 Assuming that the blood culture criteria was the best reference standard, we finally determined  
237 clinical performance of PCR assays. Both tests showed similar specificity  $> 90\%$  and Post-test  
238 probabilities associated with large or moderate usefulness of likelihood ratios ( $LR+ > 9.8$  and

239 LR- < 0.2) but significant difference in specificity (92.9% vs 82.6% for multiplex PCR and Nga-  
240 PCR respectively).

#### 241 Impact of differences in blood volumes used for testing

242 Regardless of the age group, the average volume used for PCR (3.4mL) was similar (Figure 1a),  
243 including 11.6% cases with a volume  $\leq$  3.0 mL. In contrast, a significant larger blood volume in  
244 average was used for culture in adults (3.5mL) than in both groups of children  $\leq$  17 years (2.3  
245 mL) (Figure 1b), including 385/549 (70.1%) cases with a blood volume  $\leq$  3 mL. In absence of  
246 significant difference in volume used for either PCR or culture among groups of patients  $\leq$  17  
247 years old ( $p < 0.01$ ), we decided to merge them for further analysis. However, in order to control  
248 for potential bias due to different volumes used for PCR vs culture, we also separately analyzed  
249 all patients for which volume of PCR vs culture differed by more than -0.1 mL (Figure 1c and  
250 Table 4). There was a total of 214 cases (54% children, 46% adults) with a reported higher  
251 volume for BC (3.6 mL vs 3.5 mL) than PCR (3.3 mL vs 3.2 mL); this subset included 6 of 7  
252 culture-positive specimens that were PCR negative. Only 9 samples (4.2%) from children had a  
253 volume  $\leq$  3 mL for culture as well as for PCR. For this subset of samples, performance of the  
254 multiplex PCR demonstrated a drop in sensitivity (92.9% to 82.4%) and specificity (92.3% to  
255 90.0%) with better results observed among children (Table 4).

256 Clinical performance estimated by Latent Class Model (LCM) indicated 73% sensitivity (including  
257 69% in adults) and 99% specificity in all age groups for culture (Table 5A). For both PCR  
258 assays, > 90% sensitivity and specificity as well as LR+ >10 and LR- <0.1 in adults were  
259 reported. When assessed in absence of difference in blood volume used for testing (Table 5B),  
260 LCM estimates reported a decrease in culture sensitivity (73% to 69%) as a result of a drop in  
261 older patients (61%). In contrast, reported performance for both PCR assays indicated a raise in  
262 sensitivity in all age groups except in adults with Nga-PCR (93% to 90%). No or few variations in  
263 specificity for PCR assays were observed.

264 **DISCUSSION**

265 The assay described here was designed for the simultaneous identification and differentiation of  
266 *S. Typhi*, *S. Paratyphi A* and NTS. The molecular basis of the present assay was identified  
267 through *in silico* analysis of *Salmonella enterica* CRISPR loci polymorphism<sup>35</sup>, and consists of  
268 unique and constant spacers in *Salmonella* serovars Typhi and Paratyphi A that are suitable for  
269 distinguishing the two serovars. Analytical performance of the qualitative qPCR results yielded  
270 an  $R^2 > 0.980$  and PCR efficiency in the range of 80-120%<sup>36</sup>. The assay demonstrated robust  
271 specificity similar to others studies<sup>28,31</sup>, with no cross detection in isolates, and absence of  
272 detection in controls. The limit of detection was 10 copies/reaction, equivalent to 1 copy/ $\mu$ l when  
273 compared to final elution volume.

274 The addition of bile in culture media promoted the release of trapped bacteria in leukocytes and  
275 decreased serum bactericidal activity<sup>37</sup>. Multiplex and Nga-PCR assays detected additional *S.*  
276 Typhi/Paratyphi-A (n=45 and n=49 respectively) compared to traditional blood culture. In a  
277 recent review<sup>8</sup>, other PCR assays reported sensitivities at low (< 15%), moderate, or high ( $\geq$   
278 85%) rates in culture-negative samples. In our study, detection rates were low in culture-  
279 negatives, as low as 7.7% and 8.5% with the multiplex PCR assay and Nga-PCR assays  
280 respectively in line with reported results from large cohorts. Unfortunately, we did not perform a  
281 secondary end-point test, as initially considered, and thus do not have an independent  
282 confirmation of infection vs past exposure in the PCR positive detections among the culture-  
283 negative samples.

284 Despite good overall sensitivity of the PCR assay, we and others<sup>27,28,38</sup>, failed to detect *S.*  
285 Typhi/Paratyphi-A in 7 of the 98 culture-positive samples. Assay failure for those cases could not  
286 be attributed to sample volume used for testing, nor to the genetic diversity of the bacterial  
287 strains, because the cultured strains were subsequently identified by PCR (data not shown). The  
288 longer time to positivity in these samples suggests very low initial bacteremia and/or presence of

289 antibiotics. The commercial BC bottles contain resins that bind and neutralize antibiotics,  
290 increasing the chance of pathogen recovery (range 15-35%) in patients on antibiotic therapy  
291 compared with non resin-based culture media <sup>39</sup>. In absence of resins in the pre-enrichment  
292 media, growth of bacteria during the 5-hour incubation period might be inhibited with a bacterial  
293 inoculum below the detection limit of PCR. Moreover, considering the low median reported  
294 concentration of *Salmonella* Typhi in blood <sup>40</sup> (0.3–1 CFU/mL) and the potential low bacteremia  
295 in specimen, discrepant samples may be explained by absence of bacteria in aliquots.

296 An important limitation of this study was the generally low blood volume, particularly for adult  
297 participants, as well as the difference in blood volume used for culture (average 2.5mL) and  
298 PCR (average 3.4mL). The use of a limited volume of blood (2 to 3 mL) for cultures from  
299 children is a current practice despite better bacterial growth with large (> 5 mL) volume of blood  
300 <sup>32</sup>. In addition, while high and repeated volumes are associated with higher probability of  
301 positivity in culture <sup>9</sup>, none of the 13 positive cultures in adults have been performed with more  
302 than 4 mL of blood. However, despite assumed suboptimal conditions, culture results relative to  
303 PCR indicated 67% sensitivity in the overall population (supplementary table 2), in line with data  
304 reported in a recent review <sup>41</sup>. The issue of blood volume and culture sensitivity underscore the  
305 need for newer, more sensitive assays for *Salmonella* that can perform at these lower volumes,  
306 highlighting the rationale for studies such as ours. Thus, the proportion of positive blood cultures  
307 was not significantly different (chi-square test) depending on whether culture was performed on  
308 < 2 mL (28/187), 2 to 3 mL (31/217) or 3 to 4 mL (38/253) of blood nor related to age (85/542 in  
309 children < 17 years old and 13/138 in adults).

310 Because sites with high typhoid endemicity are the same ones where obtaining blood specimens  
311 at higher volumes are a challenge, blood culture remains a poor performer under real world  
312 conditions and could not be considered as a satisfactory gold standard. Latent class analysis is  
313 a model-based approach to approximate the sensitivities and specificities of different tests in

314 absence of reliable gold standard<sup>42</sup>. Thus, LCM considers that each test could be imperfect in  
315 diagnosing the true disease status and estimates which of these assays are the best performers  
316 under conditions and limitations of this study. The true disease status of the patient population is  
317 then defined on the basis of overall prevalence (the probability that a patient with suspected  
318 typhoid fever is truly infected with *S. Typhi*). LCM indicates 73% sensitivity in overall population  
319 (74% in children under 17 years old), in the high range of reported estimates <sup>10</sup>. However, in  
320 absence of secondary end point test to confirm PCR results, there is a non-neglectable risk of  
321 class membership misclassification (confirmed/suspected) in addition to the unbalanced weight  
322 of the respective tests. This might be counterbalanced by running the model with results from  
323 bone marrow culture and/or serological tests results.

324 Despite short turnaround times, additional sensitivity benefit and capacity to serotype, the utility  
325 of PCR-based tests for enteric disease detection is still under debate. This study provided  
326 clinical performance results for a new assay that combined culture incubation steps with  
327 multiplex real-time PCR. Further evaluations are needed to compare performance of the assay  
328 relative to bone marrow culture as well as to repeat clinical evaluation in other geographical  
329 areas where NTS are more prevalent. Our multiplex PCR assay demonstrated robust  
330 improvements in detection over blood culture, and performance was best in children for which  
331 blood sample volumes were limited. Additional developments have made it possible to market a  
332 ready-to-use PCR kit (freeze-dried) that uses a larger volume of input DNA extract (15 µL). As  
333 molecular diagnostics are becoming increasingly accessible in developing country clinical  
334 laboratories, this new tool may be useful alone or in combination with other assays, particularly  
335 in the context of high disease burden, and for use in epidemiological studies and as secondary  
336 endpoint test for future vaccine trials.

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344 **Authors' current addresses :**

345 **Stephane Pouzol:** Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre  
346 International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de  
347 Lyon, UCBL1, Lyon, France. [stephane.pouzol@fondation-merieux.org](mailto:stephane.pouzol@fondation-merieux.org)

348 **Arif Mohammad Tanmoy:** Child Health Research Foundation (CHRF), Dhaka, Bangladesh.  
349 [arif.tanmoy@gmail.com](mailto:arif.tanmoy@gmail.com)

350 **Dilruba Ahmed:** International Centre for Diarrhoeal Disease Research (icddr,b) Dhaka,  
351 Bangladesh. [dahmed@icddr.org](mailto:dahmed@icddr.org)

352 **Farhana Khanam:** International Centre for Diarrhoeal Disease Research (icddr,b) Dhaka,  
353 Bangladesh. [farhanak@icddr.org](mailto:farhanak@icddr.org)

354 **W. Abdullah Brooks:** Bloomberg School of Public Health, Department of International Health,  
355 Johns Hopkins University, Baltimore, MD, USA. [abrooks@mac.com](mailto:abrooks@mac.com)

356 **Golam Sarower Bhuyan:** Institute for Developing Science and Health Initiatives (ideSHi),  
357 Dhaka, Bangladesh. [sarower@ideshi.org](mailto:sarower@ideshi.org)

358 **Laetitia Fabre<sup>6</sup>:** Institut Pasteur, Unité des Bactéries Pathogènes Entériques, Paris, France.  
359 [laetitia.fabre@pasteur.fr](mailto:laetitia.fabre@pasteur.fr)

360 **Juliet E Bryant:** Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre  
361 International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de  
362 Lyon, UCBL1, Lyon, France. [juliet.bryant@fondation-merieux.org](mailto:juliet.bryant@fondation-merieux.org)

363 **Marie-Paule Gustin** : Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre  
364 International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de  
365 Lyon, UCBL1, Lyon, France and Institute of Pharmacy, Department of Public Health, University  
366 of Lyon 1, Lyon, France. [MARIE-PAULE.gustin@univ-lyon1.fr](mailto:MARIE-PAULE.gustin@univ-lyon1.fr)

367 **Philippe Vanhems** : Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre  
368 International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de  
369 Lyon, UCBL1, Lyon, France and Service d'Hygiène, Epidémiologie et Prévention, Hôpital  
370 Edouard Herriot, Hospices Civils de Lyon, Lyon, France. [philippe.vanhems@chu-lyon.fr](mailto:philippe.vanhems@chu-lyon.fr)

371 **Bill Carman** : Fast Track Diagnostics, Esch sur alzette, Luxembourg. [Bill.Carman@fast-](mailto:Bill.Carman@fast-trackdiagnostics.com)  
372 [trackdiagnostics.com](http://trackdiagnostics.com)

373 **François-Xavier Weill** : Institut Pasteur, Unité des Bactéries Pathogènes Entériques, Paris,  
374 France. [francois-xavier.weill@pasteur.fr](mailto:francois-xavier.weill@pasteur.fr)

375 **Firdausi Qadri**: International Centre for Diarrhoeal Disease Research (icddr,b) Dhaka,  
376 Bangladesh. [fgadri@icddr.org](mailto:fgadri@icddr.org)

377 **Samir Saha**: Child Health Research Foundation (CHRF), Dhaka, Bangladesh.  
378 [samir@chrfd.org](mailto:samir@chrfd.org)

379 **Hubert Endtz** Laboratoire des Pathogènes Emergents, Fondation Mérieux, Centre International  
380 de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS, UMR5308, ENS de Lyon,  
381 UCBL1, Lyon, France and Department of Medical Microbiology and Infectious Diseases,  
382 Erasmus MC, Rotterdam, The Netherlands. [Hubert.ENDTZ@fondation-merieux.org](mailto:Hubert.ENDTZ@fondation-merieux.org)

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525 Chittagong, Bangladesh. *Trop Med Int Health*. 2015;20(10):1376-1384.  
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