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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Design of the molecular assay

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

Real-time PCR Method

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

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

In vitro performance of the molecular assay

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

147 *Salmonella* variation as previously described [35] : 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 Icddr,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

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

Clinical Study Laboratory Method

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

Ethics

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

Statistical analysis

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

RESULTS

In vitro performance of the multiplex real-time PCR assay

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

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

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

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

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

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

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

Impact of differences in blood volumes used for testing

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

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

DISCUSSION

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

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

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

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

An important limitation of this study was the generally low blood volume, particularly for adult participants, as well as the difference in blood volume used for culture (average 2.5mL) and PCR (average 3.4mL). The use of a limited volume of blood (2 to 3 mL) for cultures from children is a current practice despite better bacterial growth with large (> 5 mL) volume of blood [32]. In addition, while high and repeated volumes are associated with higher probability of positivity in culture [9], none of the 13 positive cultures in adults have been performed with more than 4 mL of blood. However, despite assumed suboptimal conditions, culture results relative to PCR indicated 67% sensitivity in the overall population (supplementary table 2), in line with data reported in a recent review [41]. The issue of blood volume and culture sensitivity underscore the need for newer, more sensitive assays for *Salmonella* that can perform at these lower volumes, highlighting the rationale for studies such as ours. Thus, the proportion of positive blood cultures was not significantly different (chi-square test) depending on whether culture was performed on < 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 children < 17 years old and 13/138 in adults).

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

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

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

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