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A novel multiplex real-time PCR for the detection of *Salmonella Typhi*, *Salmonella Paratyphi* A and *Burkholderia pseudomallei* in clinical samples

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Abstract

Enteric fever, caused by infection with Salmonella Typhi or Paratyphi A, B, or C (typhoidal Salmonella) and melioidosis are among the most common bacterial causes of acute febrile illness in tropical and subtropical countries. The diseases are widely spread even in developed countries mainly affecting travellers returning from endemic areas. Melioidosis is an increasingly recognized fatal septicemic infection also mimicking tuberculosis. These pathogens are largely underreported due to lack of sensitive and specific diagnostic tools. In this study, an inhouse multiplex real-time PCR assay was developed for the simultaneous detection of *Salmonella Typhi*, *S. Paratyphi* A and *B. pseudomallei* using specific primers. The inhouse developed assay was evaluated on buffy coat and serum samples collected from patients with acute febrile illness at four different centres. The assay had a detection limit of less than 1 genome copy for *S. Typhi* and *S. Paratyphi* A and 18 genome copies per 10 µl of PCR input for *B. pseudomallei*. Among the 1101 samples tested by multiplex real-time PCR, one (0.09%) sample was positive for *S. Typhi*. One sample was positive for blood culture identified as *S. Typhi* but negative by real-time PCR. The samples were negative for *S. Paratyphi* A and *B. pseudomallei*. The multiplex real-time assay would be highly useful as a diagnostic aid for the syndromic approach and comprehensive diagnosis of enteric fever and melioidosis. The assay could improve the overall diagnostic capability and be a useful tool during outbreak investigations.

Keywords: acute febrile illness; enteric fever; melioidosis; multiplex real-time PCR;

Introduction

Acute febrile illness is a common presentation in many healthcare setting and the etiological agents are very diverse.

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Early and reliable detection is an essential step in the management of infectious diseases. The timely diagnosis and treatment are trivial due to lack of rapid, sensitive and specific diagnostic tools resulting in presumptive diagnosis and empirical treatment with antimicrobials. Early detection would help provide appropriate patient care, prevent outbreaks, and determine treatment outcome [1]. In syndromic diagnosis, where different pathogens present with similar symptoms, the use of highly sensitive and

specific diagnostic methods would help in understanding the real spectrum of a particular disease in the specific geographic population [2]. Pathogens such as Salmonella Typhi, Salmonella Paratyphi A and Burkholderia pseudomallei are the important causes of acute febrile illness especially in tropical and subtropical countries [3,4]. These pathogens contribute to the global burden with a high incidence rate in South Asia, sub-Saharan Africa, East Asia and Pacific. High rates of newly diagnosed cases are reported in Regions such as North America and Europe and Central Asia through travel from endemic areas [5]. This indicates the importance of early detection and prompt treatment. Multiplex assays are now considered an appropriate clinical diagnostic application especially when the identification of etiological agent is challenging [6]. The study was aimed to develop an inhouse multiplex real-time PCR for the detection of the three important pathogens and to look at the frequency of these pathogens in patients presenting with acute febrile illness.

Materials and Methods

In this study, a multiplex real-time PCR assay was developed and evaluated on clinical samples collected from patients with acute febrile illness.

Inhouse multiplex real-time PCR

The multiplex real-time PCR cocktail contained primers and probes specific for S. Typhi, S. Paratyphi A and B. pseudomallei (Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as internal amplification control. The target genes for PCR amplification were selected based on previous literature that has demonstrated high sensitivity and specificity in clinical samples. Species-specific primers and probes were designed by using IDT oligo design software. The identified primer and probe sequences were cross-checked for specificity by BLAST analysis. Primers and probes were obtained from commercial sources (Eurofins, India). Upon receipt, the lyophilized primers and probes were resuspended in TE buffer (10 mM Tris•Cl, 1 mM EDTA, pH 8.0) to provide a stock solution of 100 µM, and divided into small aliquots to avoid repeated freeze-thaw.

Table 1: List of primers and probes used in the study

Pathogens	Forward primer^	Reverse Primer^	Probe^
Salmonella Typhi	GCTCTAAAACACTGGGACTTG	TCTGGGCTGTAATAGGATCTG	6-FAM - TGTAGGCATCTTGGA CATTAAGC - BHQ1
Salmonella Paratyphi A	GTCAGGATCAGTAAGCATGTG	TCCTTGCCGTATTTTCTATGC	HEX - AGTCATTTTCCGTAT AAACTCCACC - BHQ1
Burkholderia pseudomallei	TCAACCCCATGAGATCGAG	GCAAATGGTCACGAAGCTC	CY5 - ATTTCGCCTTCGGAT TCGC - BHQ2
GAPDH[#]	GGAAACTGTGGCGTGATG	CGTTCAGCTCAGGGATG	TexasRed - AGTAGAGGCAGGGA TGATGTT - BHQ2

#Internal amplification control^The oligonucleotide sequences are given as 5' to 3' position. FAM, HEX, Cy5 & texas red are the fluorescent dyes; BHQ1 & 2 are quencher dyes

S. Typhi: *Salmonella enterica* subsp. enterica serovar Typhi strain encoding FliC region; NCBI accession No. CP029646; nucleotide position: 1965599 to 1965718.

S. Paratyphi: *Salmonella enterica* subsp. enterica serovar Paratyphi A strain encoding a hypothetical protein; NCBI accession No. FM200053; nucleotide position: 2572476 to 2572583

B. pseudomallei: *Burkholderia pseudomallei* MSHR305 chromosome 2 encoding epaR gene, a type III secretion apparatus protein; NCBI accession No. CP006469; nucleotide position: 1520940 to 1521049

GAPDH: Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1, mRNA; NCBI accession No. NM_001289745.2; nucleotide position: 746 to 844.

Synthetic DNA constructs of 250 basepairs harbouring the PCR amplification region cloned in a suitable plasmid vector were commercially synthesized and obtained (Eurofins, India). The DNA constructs for all the three pathogens and the housekeeping gene were used to ascertain the lower limit of detection (assay sensitivity). The synthetic DNA templates were also used as a positive control in all the real-time PCR assays. In the real-time PCR setup for patient sample testing, a negative control was included as every third sample by replacing DNA template with nuclease-free water. The functionality of each set of primers and probe were checked in uniplex PCR assays against cloned gene constructs before combining the different sets in a multiplex PCR assay and the same were optimized for high specificity and sensitivity of detection. The real-time multiplex PCR amplification was performed as 25µl reactions using by QuantiTect Multiplex PCR NoROX master mix (Qiagen, Hamburg, Germany) in Rotor-Gene Q 5-plex System (Qiagen, GmbH, Germany). DNA extract (10µl), forward and reverse primer at a final concentration of 1.5 picomoles each and probe at a final concentration of 0.5 picomoles. The thermal profile includes initial activation at 95°C for 15 min followed by 45 cycles of Denaturation at 94°C for 60 sec and annealing/extension at 60°C for 60 sec. Amplification in a typical sigmoid curve within the 40^{th} cycle with a minimum of 0.05 fluorescence intensity was considered as the cut-off for real-time PCR as previously described (Herthnek and Bölske, 2006). The samples that showed amplification after the 35^{th} cycle were retested in uniplex format. Those samples that were positive on retesting alone were considered as true positives.

Establishment of the lower limit of detection

The cloned plasmids were serially diluted down 10-fold in TE buffer (pH 8.0) (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) within the concentration range of 10^{-1} to 10^{-12}. Each dilution was tested in triplicates by the inhouse real-time PCR in uniplex format individually for each target gene. Appropriate negative controls were included. Amplification shown in the highest dilution (least concentration) in at least two of the triplicates tested on each dilution was taken as the lower limit of detection and expressed as plasmid copies per microliter.

Subject recruitment and sample collection

Patients presenting with acute febrile illness were recruited from Sri Narayani Hospital and Research Centre (SNHRC), a 300-bed multispecialty hospital serving in the rural area of Vellore district, South India. Blood samples were collected from patients after obtaining informed consent/assent in English or vernacular language. A detailed clinical proforma was obtained from each patient during the sample collection. Ethical approval from the Institutional Review Board has been obtained for the study (IEC/IRB No: 27/16/09/13). Samples from a total of 536 patients were collected and separated into two parts. One part was used for blood culture in BacTAlert system and the other was collected in a vacutainer containing EDTA. Blood culture bottles flagging positive signals were processed for bacterial identification and antimicrobial susceptibility testing using standard methods [7]. The blood culture remained as the gold standard for the three pathogens tested. Buffy coat was separated from the EDTA blood as described previously (Nandagopal et al. 2010). Buffy coat was suspended in phosphate buffer saline (pH 7.3) and plasma samples were stored at -20°C until use. Blood or serum samples collected from patients with acute febrile illness collected from three other collaborating institutions were also tested. The three centres include (1) an upgraded primary health centre (PHC), Ussoor, Vellore, (2) a medical research institute and reference centre called King Institute of Preventive Medicine (KIPM), Chennai, Tamil Nadu and (3) a tertiary care medical college, Pushpagiri Institute of Medical Sciences (PIMS), Thiruvalla, Kerala. Blood culture was carried out for all the samples obtained from the parent study centre (SNHRC). The blood samples obtained from PHC was available only for buffy coat preparation and the other two collaborating institutions shared only serum samples. The inhouse multiplex real-time PCR testing was carried out on all samples (n=1101) and rapid card IgG/IgM anti-*S. Typhi* and anti-*S. Paratyphi* testing was done on random samples (n=178) (Table 2).

Table 2: Samples tested from four different study centres

Study centres	Samples collected (n)	Nature of sample	Blood culture	Real-time PCR	TyphiDot on random samples (n=178)
Sri Narayani Hospital and Research Centre	536	Whole blood*	1	-	10/143 IgG and IgM 1/143 IgM
Upgraded PHC, Ussoor	184	Whole blood*	-	-	ND
King Institute of Preventive Medicine and Research	120	Serum	-	1	0/27
Pushpagiri Institute of Medical Sciences	261	Serum	-	-	0/8

*Buffy coat was separated from the whole blood and used for PCR; ND - not done.

Real-time PCR testing on clinical samples

DNA was extracted from buffy coat/serum using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. During the DNA extraction setup, every fifth sample acted as an extraction control replacing the clinical sample with PCR-grade nuclease-free water. This was carried out to ensure the absence of inhibitory substances on extraction. A total of 1101 samples were collected from all the four centres as either whole blood or serum. The samples were tested for the three pathogens by the inhouse developed multiplex real-time PCR. The housekeeping gene, GAPDH was included in the cocktail and acted as internal amplification control. DNA samples extracted from standard strains (S. Typhi, S. Paratyphi A, Burkholderia cepacia) and an inhouse B. pseudomallei strain were tested for proficiency testing. For heterologous testing, DNA extracted from bacterial cultures including, Escherichia coli, Pseudomonas species, Klebsiella species were prepared and tested by the multiplex real-time PCR assay.

Test for IgG / IgM typhoid fever: From the overall samples (n=1101), a set of random samples (n=178) were generated. These samples along with two samples positive for Salmonella by blood culture were tested for IgM and IgG anti - S. Typhi and S. Paratyphi using OnSite Typhoid IgG/IgM rapid lateral flow immunoassay test kits (CTK Biotech Inc, California, USA) according to manufacturer's instructions.

Results

An inhouse multiplex real-time PCR for the simultaneous detection of S. Typhi, S. Paratyphi A and B. psedomallei was developed. The primers and probes were found to be highly (100%) homologous and specific to each target pathogen as identified by BLAST analysis. The inhouse multiplex real-time PCR testing was carried out on all samples (n=1101) and rapid card IgG/IgM anti-S. Typhi and anti-S. Paratyphi testing was done on random samples (n=178).The samples from different areas were recruited to look at the frequency of enteric fever and melioidosis in patients presenting with acute febrile illness. This is a prospective study and cross-sectional in nature. The parent study centre, SNHRC mainly serves rural and peri-urban areas of Vellore district, India and the PHC serves rural and tribal areas of Vellore district. The KIPM centre is a regional node and a state reference laboratory in Chennai, India. Serum samples received from patients with acute febrile illness for routine serological diagnostics were randomly picked and shared for this study. At PIMS, samples were collected from patients during the monsoon and post monsoon floods of August 2018 in Kerala.The inhouse developed multiplex real-time PCR assay showed specific amplification for the three pathogens in the respective channels. Standard strains and inhouse strains also tested positive for the respective pathogens in the appropriate channels. The detection limit for each target pathogen in the real-time PCR assay was determined. The in-house real-time PCR assay had a detection limit of less than 1 genome copy per 10 µl of PCR input for S. Typhi and S. Paratyphi A, and 18 genome copies per 10 µl of PCR input for B. pseudomallei, indicating a high assay sensitivity. The detection limit for GAPDH was 43 genome copies per 10 µl of PCR input. In the heterologous testing, the multiplex real-time PCR did not amplify other pathogens indicating high specificity.A total of 1101 samples as blood or serum were collected from patients presenting with acute febrile illness from different centres. Of these, males were 645 and females were 436 (details of 20 patients were not available) and age ranged from 12 days to 92 years (median, 44). Of 536 samples collected from SNHRC for which blood culture data was available, 106 samples flagged positive and upon further testing, coagulase-negative Staphylococcus (n=55) was predominant followed by Escherichia coli (n=16) and Klebsiella species (n=6). Blood culture was negative for S. Paratyphi A and B. pseudomallei. Among the 1101 samples tested by multiplex real-time PCR, one (0.09%) sample was positive for S. Typhi (Fig. 1). This sample from KIPM was obtained as a serum sample. Among 536 samples, for which blood culture data was available, one sample positive in blood culture was identified as S. Typhi based on culture and sero-agglutination and one other blood culture positive sample was identified as non-typhoidal salmonella. Both the samples were negative by real-time PCR. The samples were negative in blood culture for S. Paratyphi A and B. pseudomallei.

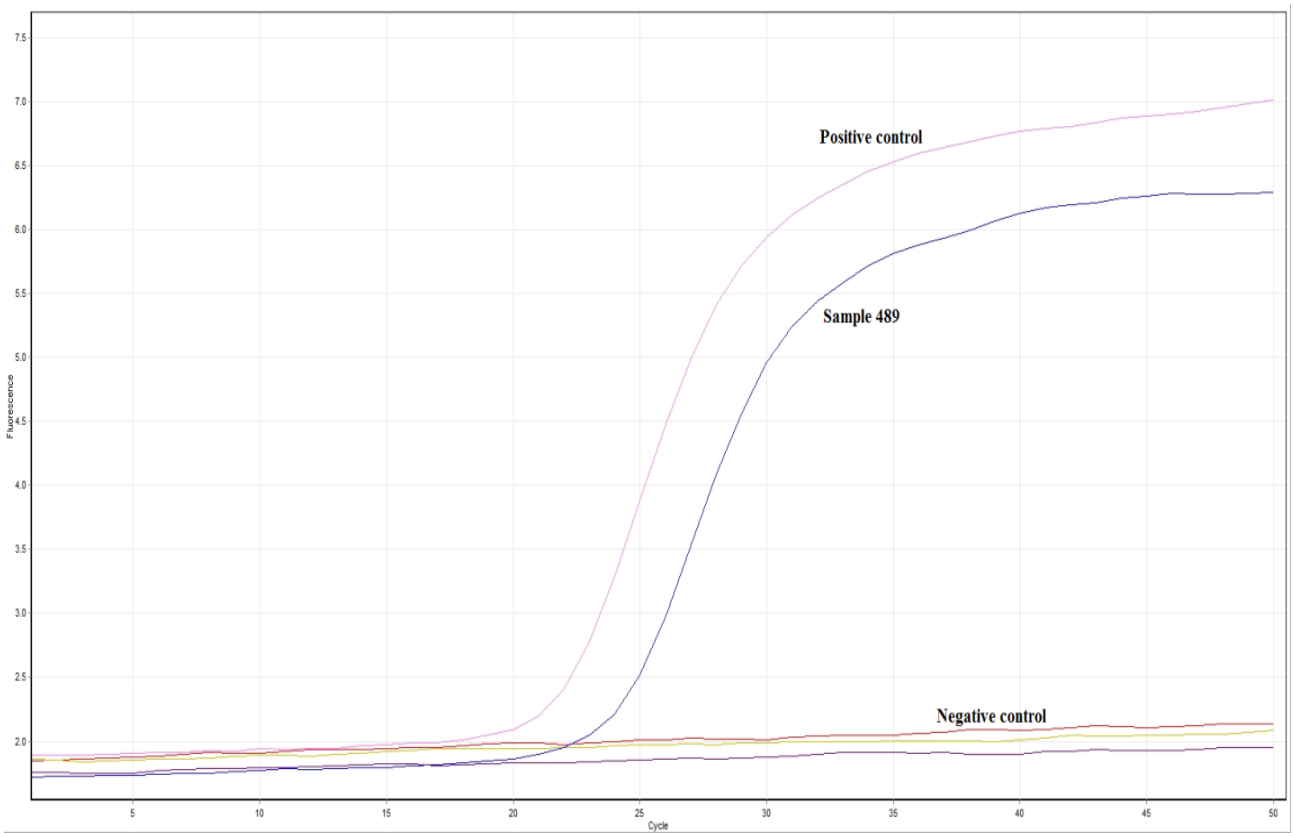

Fig 1:The real-time PCR amplification curve for a sample positive for *S. Typhi*

The major clinical presentation among the patients were gastrointestinal symptoms (40%) including diarrhea, nausea, vomiting, abdominal pain, abdominal tenderness, hepatomegaly and hepatosplenomegaly. Mild fever (< 100°F) was recorded in 220 (41%) patients, moderate fever (100 - 102°F) was recorded in 273 (51%) patients and high-grade fever (>102°F) was recorded in 33 (6.2%) patients. Majority of them had an undulating fever (88.5%) and a few had prolonged fever (8.5%). About 3% of the patients reported with rashes
(erythematous/macular/maculopapular/purpuric). Other important clinical features such as myalgia, cough, hematuria, loss of weight, co-morbidities such as diabetes, hypertension and renal disease were also recorded.

Samples (n=46) among 536 samples collected at SNHRC, were tested for Widal as part of routine investigations. Of these, 33 samples were positive. Fourteen patients had 1:80 titre for both STO and STH and five patients had 1:160 titre for both STO and STH. Eleven patients had a titre of 1:160 for STO and 1:80 for STH; and three patients had 1:80 for STO only. The sample positive for culture had 1: 160 titre for STO and STH.Among the 180 random samples tested for IgG/IgM-anti-*S. Typhi* and *S. Paratyphi* A, 10 samples were positive for both IgG and IgM and one sample was positive for only IgM. Among the samples tested, an additional 12 samples including the sample that was *S. Typhi* positive by blood culture, produced a faint band for IgG and IgM and one additional sample produced a faint band for IgM and these 13 were not considered as confirmed positive. The other sample that was identified as non-typhoidal salmonella was negative by TyphiDot.

Discussion

An inhouse multiplex real-time PCR was developed for the detection of three pathogens, *S. Typhi*, *S. Paratyphi* A and *B. pseudomallei* and evaluated in patients with acute febrile illness. Typhoid and paratyphoid fever commonly called as enteric fever, is caused by *Salmonella enterica* serovar Typhi (*S. Typhi*) and *Salmonella enterica* serovar Paratyphi (*S. Paratyphi*). A systematic analysis for the global burden of diseases in the year 2017 reported 14.3 million cases of typhoid

and paratyphoid fevers with a case fatality of 0·95% [8]. The rates are estimated to be higher in children and among the elderly population. Typhoid fever is an acute and life-threatening systemic infection with significant morbidity and mortality especially in developing countries where there is limited access to safe drinking water, good sanitation and hygienic practices [9]. The infection mostly present with protean manifestations but commonly with high-grade fever, headache, malaise and gastrointestinal symptoms (abdominal pain, vomiting, diarrhea), hepatomegaly and splenomegaly [10]. Culture from the body fluids remains the gold standard for the diagnosis, of which bone marrow culture is considered superior. Due to the unpleasant invasive procedure of collecting bone marrow, blood culture is preferred. For the definite diagnosis of typhoid fever, the sensitivity of the blood culture compared to bone marrow culture was found to be 66% and hence estimation of disease burden based on blood culture will be an underestimate [11]. Apart from the sample volume, several factors such as patients' duration of fever, prior antimicrobial use and laboratory conditions are known to highly influence the sensitivity of the blood culture [12]. These factors along with low bacterial counts in the blood and nonspecific clinical features complicate the clinical diagnosis. Widal test based on serology is used commonly in spite of its limitations in sensitivity because of ease in use and cost-effectiveness. There are both technical and clinical drawbacks in testing both acute and convalescent-phase serum samples collected one week apart and looking for an increase in antibody titre for the appropriate interpretation. In a meta-analysis on the diagnostic accuracy of different commercially available rapid diagnostic tests such as TyphiDot, an average sensitivity of 78% and specificity of 87% were observed with no difference in false positive rates [13]. In our study, of the 178 random samples tested for rapid card IgG/IgM-anti-*S. Typhi* and *S. Paratyphi* A, 10 samples were positive for both IgG and IgM and one sample was positive for only IgM. Additional 12 samples produced a faint band for IgG and IgM were not considered as true positives. The sample that was identified as S. Typhi from blood culture gave faint bands for both IgM and IgG and the sample that was identified as non-typhoidal Salmonella was negative for both IgM and IgG. In serological diagnosis, IgM is expected in the acute phase of illness and IgG in past infection. The rapid card devices have low sensitivity and specificity albeit it is easy to use. It is important for the clinicians to be cautious and not have too much of dependence on these devices for diagnosis of enteric fever which could lead to erroneous diagnosis and use of unnecessary antibiotics.

B. pseudomallei causes melioidosis and is endemic in most parts of southeast Asia with a fatality rate of up to 30% [14]. It generally presents with fever, abscess, acute pneumonia and septicemia and is called a great imitator of tuberculosis [15]. Rapid detection of *B. pseudomallei* would help initiate early antimicrobial therapy that could significantly improve patient outcome. While blood culture is the standard approach, the low detectable limit of the pathogen in the blood (~1 CFU/mL) hampers the timely diagnosis and treatment [16]. The isolation rate of enteric fever and melioidosis is generally low and therefore in our study, samples from multiple sampling sites were chosen and tested. Acute febrile illness is caused by a wide range of pathogens and is common in tropical and subtropical countries. The traditional diagnostic approach of blood culture is time-consuming and the reliance on IgM/IgG testing poses a risk of false positives. The automated identification system is still beyond reach. In a recent multisite surveillance study, a total of 267536 blood cultures were retrospectively analyzed and 0.53% of which was positive for *S. Typhi* and *S. Paratyphi* indicating a relatively low isolation rate of *S. Typhi* in India [17]. In our study, among blood culture positives in 536 samples, we found two cultures each identified as *S. Typhi* and non-typhoidal Salmonella. In real-time PCR, one sample was identified from a serum sample.

Molecular detection by PCR is generally considered a rapid and highly sensitive and specific approach for the detection of pathogens especially the uncultivable or undetectable etiological agents. *S. Typhi*, *S. Paratyphi* A and *B. pseudomallei* being potential agents of acute febrile illness and not detectable in conventional culture techniques due to various reasons, we developed a multiplex real-time PCR for the detection of these pathogens and evaluated in patients with acute febrile illness. A multiplex PCR for the simultaneous detection of Burkholderia species have been reported previously [18–20] but was not evaluated in clinical samples. Other studies have reported multiplex PCR for the detection of *B. pseudomallei* along with Brucella species, *Bacillus anthracis* and *Yersinia pestis* [21] but the assay was not evaluated in clinical samples. We previously showed the usefulness of multiplex PCR for the detection of *B. pseudomallei* along with *S. Typhi* and *Mycobacterium tuberculosis* in the buffy coat samples compared to blood culture [22]. The routine blood culture was able to identify one extra typhoid case compared with real-time PCR. While both whole blood and buffy coat are proved to be a useful clinical specimen for the detection of *S. Typhi*, the recovery rate from these samples might differ due to a number of reasons. It has been shown that the varied *S. Typhi* count in the whole blood of patients could be

attributed to the disease severity, duration of illness and patient's age. The viable pathogens, expressed as CFU, were found to be spread more in the buffy coat layer (62.5%) compared to erythrocyte and plasma layers (37.5%). The *S. Typhi* count in the whole blood was found to be three times higher in children less than 15 years of age compared to adults [23].

PCR negativity in the one culture-positive for *S. Typhi* may be attributed to following reasons. Widal at 1:160 titre (for STO and STH) and TyphiDot positivity suggested an active infection. Though PCR detection limit was less than one genome copy, the determination was not performed in spiked clinical sample but in titrated plasmid dilutions. PCR inhibitors are a possible cause [24]. But the possibility of PCR inhibitors were ruled out because the amplification of internal amplification control (GAPDH) was satisfactory. Blood culture is generally incubated for up to seven days. The blood culture in this case was positive after 36 hours of incubation. This would have enhanced the bacterial growth from the sample with possibly a very low bacterial load. While in PCR, the input could be low since buffy coat was extracted on day-one without enrichment of bacterial cells. The other possibility is "poor yield of buffy coat and thereby insufficient DNA quantity" from the patient's blood sample. The blood sample was drawn for both blood culture and buffy coat separation at the same time, so blood volume for buffy coat preparation might have been compromised. From our previous experience, buffy coat separated from 5mL of blood was sufficient for all PCR assays [22,25,26].Our inhouse multiplex real-time PCR assay was able to identify the inhouse standard strains, *S. Typhi*, *S. Paratyphi* A and *B. pseudomallei* efficiently and did not amplify other pathogens during heterologous pathogens testing. This indicates its high assay specificity. The lower limit of detection experiment indicated that the multiplex real-time PCR assay has high sensitivity. It is imperative that the real-time PCR assay along with blood culture provides the benefits of increased diagnostic yield. The assay could thus help improve laboratory diagnosis in high-incidence settings especially when enteric fever and melioidosis is suspected. The one real-time PCR positive was from serum sample and blood culture data was not available. The number of blood cultures positive were low and therefore diagnostic accuracy indices were not calculated. Despite these limitations, we recommend the real-time PCR assay for its multiplexing capability, lower detection limit and short turnaround time.In the present study, the multiplex real-time PCR was developed targeting specific genes that are suitable for the multiplex format. The inhouse assay was highly sensitive in terms of lower detection limit down to less than 1 genome copy for *S. Typhi* and *S. Paratyphi* A and 1.8 genome copies for *B. pseudomallei* per microlitre of PCR input. The assay also showed specific amplification with inhouse standard strains with no cross-reactivity with other related pathogens indicating a high specificity. The assay thus could be highly useful as a diagnostic aid for the syndromic approach and comprehensive diagnosis of enteric fever and melioidosis. Such PCR assays could help rapidly diagnose during an outbreak situation and initiate appropriate patient management. The assay could improve the overall diagnostic capability and disease surveillance.

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