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ORIGINAL RESEARCH ARTICLE

A Multiplex Nested PCR for the Simultaneous Detection of *Salmonella typhi*, *Mycobacterium tuberculosis*, and *Burkholderia pseudomallei* in Patients with Pyrexia of Unknown Origin (PUO) in Vellore, South India

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Abstract

Background and Objectives *Salmonella typhi*, *Mycobacterium tuberculosis*, and *Burkholderia pseudomallei* are among the most important monocyte-tropic bacterial agents causing pyrexia of unknown origin (PUO), with a significant number of endemic infections in both South and Southeast Asian regions. These infections pose a major risk to travelers to these regions as well.

Methods We developed and evaluated a multiplex nested polymerase chain reaction (PCR) for the simultaneous detection of the three pathogens in 305 patients' buffy coat samples.

Results The assay for *S. typhi* and *B. pseudomallei* was able to detect down to 1 colony forming unit/5 μL PCR input and *M. tuberculosis* was detected down to 20 genome copies/5 μL PCR input. *S. typhi* was detected in 10 (3.3 %) individuals, *B. pseudomallei* in 10 individuals (3.3 %), and *M. tuberculosis* in 18 individuals (5.9 %). Co-infections of *M. tuberculosis* and *B. pseudomallei* were detected in three individuals and *S. typhi* and *B. pseudomallei* in two individuals.

Conclusion This protocol is efficient for PUO diagnosis especially in Asian countries.

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1 Introduction

Pyrexia of unknown origin (PUO) is a major cause of morbidity and mortality in South and Southeast Asian countries. Many individuals present with undifferentiated fever, which is categorized as PUO pending specific investigation for tuberculosis, enteric fever, brucellosis, melioidosis (the great imitator of tuberculosis), and viral fevers [1–3]. PUO is a diagnostic challenge for clinicians; fever is a cardinal manifestation of persistent infection. Currently, in practice, PUO refers to prolonged fevers with temperatures of at least 101 °F (38.3 °C) that remain undiagnosed after routine laboratory investigation.

The World Health Organization estimates the occurrence of typhoid fever in the Asian region [4] to be 274 per 100,000 population and that of tuberculosis in Southeast Asia [5] to be 189 per 100,000 population. The prevalence of HIV seropositives among tuberculosis patients was reported to be 4 % in Southeast Asia [5], and associated risk factors. Melioidosis caused by *Burkholderia pseudomallei* is a fatal septicemic infection in humans, which can at times become chronic and manifest as abscesses, chronic pneumonia mimicking tuberculosis, and fulminant septic shock with multiple abscess in internal organs [6]. The disease can cause up to 20 % of all community-acquired sepsis in Southeast Asia. Series of melioidosis cases have been reported from South and Southeast Asia [7–15].

A multiplex nested polymerase chain reaction (PCR) assay was developed that could detect the three pathogens simultaneously from the patient's buffy coat samples. The assay was evaluated in 305 blood samples collected prospectively from individuals presenting with PUO. In addition, 50 blood and sputum samples from patients with symptoms suggestive of pulmonary tuberculosis were also tested.

2 Materials and Methods

2.1 Study Design

Ethical clearance from our Institutional Review Board was obtained. The study was cross-sectional in nature. Patients attending the out-patient department of our hospital from all age groups with a history of an acute/chronic febrile illness (temperature of 101 °F) of unknown cause for 5–15 days or more were recruited. The age of the patients recruited in the study had a range of 1–91 years and the median age was 50 years.

2.2 Blood Sample Collection and Processing

About 15 mL of venous blood was collected in 5-mL volumes in three separate containers; 5 mL for routine blood culture, 5 mL for *Mycobacterium tuberculosis* culture in their respective commercial containers. The other 5 mL was collected in a sterile tube containing EDTA for buffy coat preparation. Blood culture was performed in an automated blood culture system (BacT/ALERT 3D) according to the manufacturer's instructions. Following growth indication, the sample was subcultured on MacConkey agar and blood agar followed by a series of biochemical tests for the identification.

Buffy coat samples were collected from the blood samples and DNA was extracted in batches using the QiaAmp blood mini kit (Qiagen GmBH, Hilden, Germany) as per the manufacturer's instructions and used for multiplex nested PCR testing. All the PCR reagents including Hotstar Taq polymerase were procured from Qiagen (Hilden, Germany). Negative controls were included in every assay replacing the template with nuclease-free water (Qiagen).

2.3 Sputum Sample Collection and Processing

Sputum samples ($n = 50$) were collected in a sterile wide-mouth container with all necessary safety precautions. These samples were transported to the laboratory immediately. The sputum samples were treated with NALC-NaOH prior to inoculation in BacT/ALERT 3D MP bottles as per the manufacturer's instructions. The bottles signaling positive were sent to a commercial establishment for confirmation by AccuProbe hybridization assay specific for *M. tuberculosis* detection.

An unlinked anonymous testing for antibodies to HIV-1/HIV-2 was carried out using HIV Tri-dot rapid card (J Mitra, India) on serum samples of these 50 patients.

2.4 Development and Evaluation of Multiplex Assay

The standard strain of *Salmonella typhi* (*Salmonella enterica* serovar Typhi) was purchased from The King Institute of Preventive Medicine, Chennai, India. Standard strains of *M. tuberculosis* (H37Rv) and *B. pseudomallei* were kindly provided by Prof. Mary V Jesudason, Vellore, India. The DNA extracted from the strains was used as positive controls for PCR assays.

The primer sequences for *S. typhi*, *B. pseudomallei*, and *M. tuberculosis* were as per previous uniplex nested PCR studies [16–18] and found to have high sensitivity and specificity on buffy coat and/or sputum samples. The target regions used for *S. typhi*, *B. pseudomallei*, and *M. tuberculosis* were fliC, 16S-23S spacer region, and IS6110, respectively. The inner forward and reverse primer sequences for *B. pseudomallei* were 5′-CCTCCACCAATT GCGATGATCGTT-3′ and 5′-CAATCACAACCCGGA-TAGCTTCCAC-3′ and designed using Primer 3 software.

The cycling conditions were 95 °C for 15 min, 95 °C for 45 s, 52 °C for 40 s, 72 °C for 1 min, and 72 °C for 7 min (after the 30th cycle). A 5-μL product from the first round was used as the template for the second round. The internal round of nested PCR was carried out with the above-mentioned cycling conditions. Standard precautions were employed for PCR testing, such as flow through, separate rooms, disposable plastic ware and gloves, filter-blocked tips, and dedicated micropipettes. The thermal cycling was carried out in an Eppendorf thermal cycler (Mastercycler-personal 5332; Eppendorf AG, Hamburg, Germany). An aliquot of 5 μL of amplicon was mixed with 5 μL of 6× loading dye (Fermentas Inc., Glen Burnie, MD, USA) and analyzed by gel electrophoresis in 2 % agarose (Sigma-Aldrich Corp., St. Louis, MO, USA) prepared in Tris-Borate-EDTA buffer containing 0.5 mg/mL of ethidium bromide (Sigma-Aldrich Corp.). A 100-bp ladder as a molecular weight marker (Fermentas Inc.) was used. The gels were examined in a gel documentation system (Bangalore Genei, Bangalore, India). The nested PCR amplicon sizes for *S. typhi*, *B. pseudomallei*, and *M. tuberculosis* were 343 bp, 251 bp, and 198 bp, respectively.

2.5 Establishment of Lower Limit of Detection

The lower limit of detection was established for *S. typhi* and *B. pseudomallei* using the colony count method as described previously [19]. The conventional PCR amplified product was cloned in TOPO TA vector (Invitrogen, CA, USA) for *M. tuberculosis*. Briefly, PCR products were produced with the above-described cycling conditions with final extension of 10 min at 72 °C. PCR products were checked by agarose gel electrophoresis for a single discrete band. TOPO TA cloning kit (Invitrogen) was used to clone the PCR product as

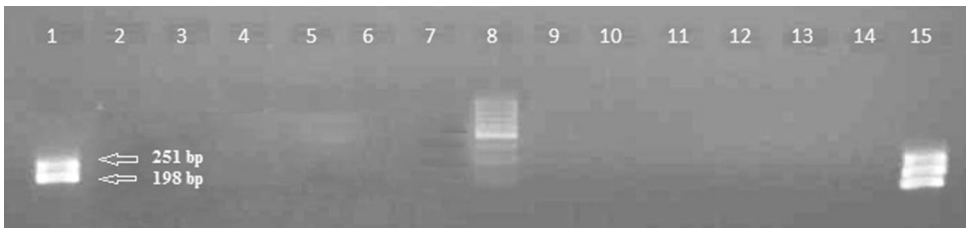

Fig. 1 A representative gel picture of patient samples positive for *Mycobacterium tuberculosis* and *Burkholderia pseudomallei*. *Lanes 1*, *2*, *4*, *5*, *7*, *9*, *11*, *12*, and *14* patient samples; *lanes 3*, *6*, *10*, and *13* negative controls; *lane 8* molecular weight marker; *lane 15* positive control

per the manufacturer's instruction. Copy number of the cloned plasmids was calculated using the formula: weight of PCR fragment (in grams per mL)/(660 g per mol = the number of base pairs of the PCR fragment) × (6.023×10^{23}) = the number of genome copies per microliter. The cloned plasmid was extracted using a plasmid extraction kit (Qiagen). The concentration of the plasmid was determined by measuring the optical density at 260 nm with an ultraviolet-visible spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, DE, USA). The cloned plasmids were serially diluted 10-fold in TE buffer (pH 8.0). The dilutions were stored at −20 °C for less than a week. The approximate number of plasmid copies/μL of DNA suspension was determined by nested PCR in triplicates using appropriate negative controls. Amplification shown in the highest dilution (least concentration) was taken as the lower limit of detection as plasmid copies per microliter. The probability of detecting bacterial DNA in a suspension of known concentration in the presence of defined DNA copy numbers was as described previously [19].

2.6 Statistical analysis

The data were entered in a MS Office 2007 Excel spreadsheet. Statistical analyses were performed using EpiInfo 6.04 Ver. Programme (CDC domain software). The Fisher exact test was used for the test of significance; p value <0.05 was considered significant. Mid-P (95 % confidence interval [CI]) was calculated in the EpiInfo statistical program.

3 Results

A total of 305 patients presenting with PUO were recruited for our study during the period from January 2011 to March 2013. Among 305 patients, 192 (63 %) were from a rural community and 113 (37 %) were from a peri-urban community, 147 were male (48.2 %) and 158 were female (51.8 %). Fever was in the range of 104–107 °F of which 4 (1.1 %) patients had hyperpyrexia (more than 105 °F) and 108 (35.4 %) patients gave a history of duration of fever more than 15 days. Of 305 patients, five died—two of them due to renal failure, one due to cardiac arrest, one due to encephalitic manifestations and, in one patient, the immediate cause of death was pulmonary embolism, this patient had an underlying malignancy.

The multiplex nested PCR showed specific amplification of *S. typhi*, *M. tuberculosis*, and *B. pseudomallei* control strains and patient samples showing co-infections (Figs. 1 and 2). The PCR was checked for individual targets and

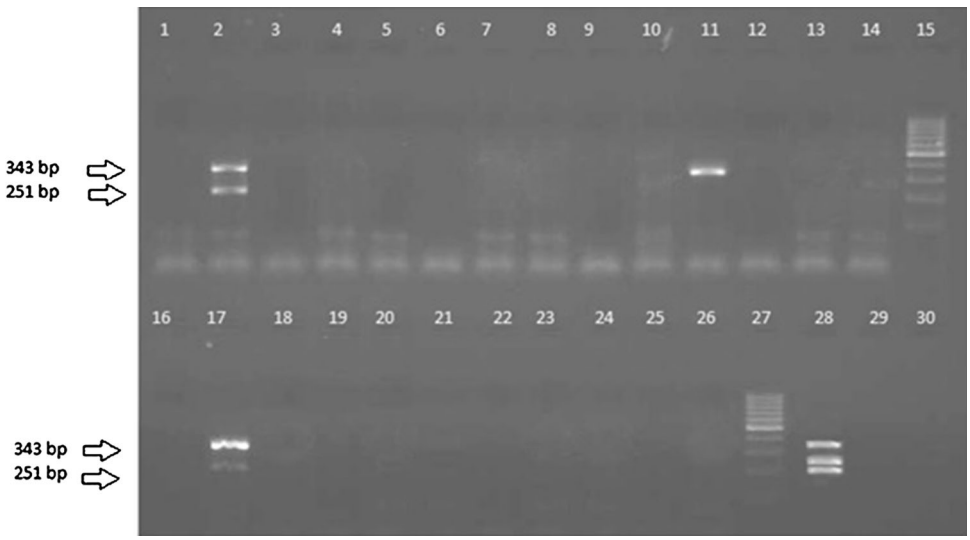

Fig. 2 A representative gel picture of patient samples positive for *Salmonella typhi* and *Burkholderia pseudomallei*. *Lanes 1*, *2*, *4*, *5*, *7*, *8*, *10*, *11*, *13*, *14*, *17*, *18*, *20*, *21*, *23*, *24*, and *26* patient samples; *lanes 3*, *6*, *9*, *12*, *16*, *19*, *22*, and *25* negative controls; *lanes 15* and *27* molecular weight marker; *lane 28* positive control; *lane 2* (sample no. 225) and lane *17* (sample no. 240): patient samples positive for *B. pseudomallei* and *S. typhi*

Table 1 Number of patients shown by sex and domiciliary status with polymerase chain reaction findings

Sex distribution and domiciliary status of the patients $n = 305$	No. of patients positive for each pathogen $n = 43$					Total positives	*P* value
	Salmonella typhi	*Burkholderia pseudomallei*	*Mycobacterium tuberculosis*	*S. typhi* and *B. pseudomallei*	*M. tuberculosis* and *B. pseudomallei*		
Male $n = 147$ (48.2)							
Rural $n = 61$ (41.5)	4	6	8	0	1	19 (44.2) [29.9–59.1]	0.04; $\chi^2 = 4.21$
Peri-urban $n = 86$ (58.5)	3	2	5	0	2	12 (27.9) [16.1–42.6]	
Females $n = 158$ (51.8)							
Rural $n = 131$ (82.9)	1	2	2	2	0	7 (16.3) [7.4–29.5]	0.72; Yates corrected $\chi^2 = 0.13$
Peri-urban $n = 27$ (17.1)	2	0	3	0	0	5 (11.6) [4.4–23.9]	

Percentages are given in parentheses. Mid-*P* values at 95 % confidence intervals are shown in square brackets

heterologous targets and found to be specific. In experiments for determination of the lower limit of detection, the assays for *S. typhi* and *B. pseudomallei* were each able to detect down to 1 colony forming unit/5 μL PCR reaction input, and for *M. tuberculosis* the assay was able to detect down to 20 genome copies/5 μL PCR reaction input.

Of 305 blood cultures from as many patients, 37 (12.1 %) grew heterologous bacteria but none of them grew *S. typhi* and *B. pseudomallei*. *M. tuberculosis* was detected in blood in seven patients. In multiplex nested PCR, 10 (3.28 %; Mid-p 95 % CI: 1.7–5.8) of 305 samples were positive for *S. typhi*; 18 (5.9 %; Mid-p 95 % CI: 3.6–8.9) of 305 samples was positive for *M. tuberculosis*, and 10 (3.28 %; Mid-p 95 % CI: 1.7–5.8) of 305 samples were positive for *B. pseudomallei*. Three (1 %) were positive for both *M. tuberculosis* and *B. pseudomallei.* Two (0.65 %) were positive for *S. typhi* and *B. pseudomallei*. In all, 43 (14.1 %; Mid-p 95 % CI: 10.5–18.3) patients were positive for any one or more of the three pathogens tested. Among 43 patients who were positive for any of the pathogen detected, the age range was 11–90 years. There were 26 male patients (60.5 %) and 17 female patients (39.5 %); 31 (72.1 %) patients were from a rural community and 12 (27.9 %) patients were from a peri-urban community. The details of the patient groups are shown in Table 1. The difference in PCR positives between rural and peri-urban male patients was found statistically significant. However, the difference between rural and peri-urban female patients was not statistically significant. The multiplex PCR results were compared with blood culture serving as the gold standard and are shown in Table 2.

Among 50 tuberculosis-suspected patients, 38 (76 %) were male, of whom 17 (44.7 %) were from a rural community and 21 (55.3 %) were from a peri-urban community; 12 (24 %) were female, of whom 8 (66.7 %) were from a rural community and 4 (33.3 %) were from a peri-urban community.

In the 50 TB-suspected patients, seven (14 %) were found to be HIV seropositive. Ten (20 %) individuals were positive for *M. tuberculosis* nested PCR, of which three were HIV seropositive. The ten positive samples were confirmed as *M. tuberculosis* by Accuprobe assay.

Table 2 Polymerase chain reaction results compared with blood culture findings

Gold standard (blood culture)	*Salmonella typhi* PCR		*Burkholderia pseudomallei* PCR		*Mycobacterium tuberculosis* PCR	
	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)
Positive	0	0	0	0	7 (2.3)	0
Negative	10 (3.28)	295 (96.72)	10 (3.28)	295 (96.72)	11 (3.6)	287 (94.1)

Generally, blood culture is used as the gold standard. However, *S. typhi* and *B. pseudomallei* did not grow in any patients' samples. Therefore, accuracy indices were not calculated. *M. tuberculosis* was positive in blood cultures in 7 of 18 polymerase chain reaction (PCR) positive samples. The accuracy indices for *M. tuberculosis* were: sensitivity of 100 % (95 % confidence interval [CI]: 56.1–100); specificity of 96.3 % (95 % CI: 93.8–98), respectively; positive predictive value of 38.9 % (18.3–63.9) and negative predictive value of 100 % (98.4–100)

4 Discussion

In this study, we developed and evaluated a multiplex nested PCR for the simultaneous detection of *S. typhi*, *B. pseudomallei*, and *M. tuberculosis* in 305 patients presenting with PUO at our hospital. *S. typhi* and *B. pseudomallei* were positive each in 3.3 % individuals, *M. tuberculosis* in 5.9 % individuals. In addition, co-infections of *M. tuberculosis* and *B. pseudomallei* and co-infections of *S. typhi* and *B. pseudomallei* were also detected.

Many infectious agents could be responsible for causing PUO and are often difficult to distinguish from one another clinically [20]. The syndromic diagnosis of PUO is cumbersome when individual tests are carried out for each specific pathogen. In a recent study by Abrahamsen et al. [21], fever etiologies in patients in South India were reported based on sputum testing, blood culture, and serological testing. They found tuberculosis to be predominant followed by other diseases including typhoid fever. In tropical countries like India, tuberculosis, typhoid fever, and melioidosis are now commonly encountered as PUO etiologies [16, 18, 22]. The literature indicates prevalence of typhoid fever, tuberculosis, and melioidosis in the same geographical areas and have frequently been reported in returning travelers to these regions as well [23, 24]. To reduce the mortality rate, early diagnosis and appropriate treatment becomes necessary. The rapid and specific diagnosis through molecular technologies like multiplex PCR can achieve specific diagnosis to allow for specific therapy. A multiplex PCR in nested format such as the one reported here will also increase the sensitivity of the assay and facilitate multiple pathogen detection in a single reaction tube.

A few investigators have developed a multiplex PCR for the detection of important pathogens causing bloodstream infections such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, Enterobacter, and Acinetobacter [25–27]. A nested PCR targeting IS6110 was found to be highly sensitive and specific in buffy coat and sputum samples [18, 20]. Melioidosis caused by *B. pseudomallei* is endemic to South and Southeast Asia. The protean presentations of melioidosis include organ abscesses, pneumonia, bacterial pneumonia, septicemia, and meningitis leading to death. Case reports on co-infection of melioidosis and tuberculosis have been reported from India [28]. A case of melioidosis masquerading as a tubercular cold abscess has also been reported [29]. Kindo et al. [30] reported a rare case of dual infection of typhoid fever and tuberculosis in a psoas abscess. Othman et al. [8] reported a case with coinfection of *S. typhi* and *B. pseudomallei* in tsunami survivors. These findings were primarily based on conventional culture. We here report three cases of co-infection of melioidosis and tuberculosis and two cases of *S. typhi* and *B. pseudomallei* in patients with acute febrile illness using multiplex nested PCR. This reflects the efficiency of our assay. *S. typhi* (14.4 %) and *B. pseudomallei* (12.6 %) were found to be the key pathogens in bloodstream infections next to *E. coli* [31].

The clinical presentation of melioidosis may mimic tuberculosis (both cause chronic suppurative lesions unresponsive to conventional antibiotics and both commonly affect the lungs) and is called “the great imitator”. Melioidosis is under diagnosed and often mistreated as tuberculosis resulting in mortality [32]. The two diseases have overlapping risk profiles (e.g., diabetes mellitus, corticosteroid use), and both *B. pseudomallei* and *M. tuberculosis* are intracellular pathogens. The site from which latent infection may reactivate as relapse after apparently successful treatment and an extended incubation period after exposure suggest a dormant state similar to that seen in tuberculosis. Case reports of melioidosis and previous or subsequent mycobacterial infection (*M. tuberculosis*, *M. terrae*) may reflect a common host susceptibility to these intracellular pathogens. All the three organisms of interest to the current research have been found to be major causes of PUO.

Melioidosis is reported to be prevalent among people involved in rice cultivation and rearing of farm animals especially in Southeast Asia where it is as common as enteric fever is in South Asia [33–35]. In our study, *B. pseudomallei* was positive in higher numbers among rural male individuals probably reflecting agricultural activity. Accurate identification of Salmonella and Burkholderia through conventional blood culture and biochemical identification is presently the yardstick for diagnosis, but the time required and frequent negative results are important limitations. *B. pseudomallei* are also shown to give false-positive Widal titers and are often misdiagnosed or underdiagnosed [36]. Even automated microbiology systems have been shown to misidentify the organism, with the identity confirmed only by molecular testing because of a high index of suspicion [37].

To date, nucleic acid amplification test evaluations have used blood culture as the gold standard for the diagnosis of typhoid fever, although bone marrow aspirate culture is recommended [38]. Bone marrow culture is highly sensitive, but is not routinely performed because of the invasiveness and technical difficulty of the procedure [39].

Blood culture provides definitive evidence of infection both in typhoid fever and melioidosis, but it fails to detect all cases, because of low bacterial load in the peripheral circulation, the presence of biological interferences present in the clinical sample that prevent growth in culture, and/or prior exposure to antibiotics [40]. The laboratory diagnosis

directly depends on the day of illness and the investigation sought. Large studies have to rely on blood culture that has sensitivity between 30 and 70 % [41]. There is no evidence to date to suggest that automated systems overcome this shortcoming where manual and automated blood cultures were equally sensitive for the detection of *S. typhi* [42]. Melioidosis has variable manifestations and many strains have ambiguous biochemical profiles, it is often difficult to have blood culture alone as the gold standard even with automated systems for phenotypic identification of *B. pseudomallei* [43].

Because of these reasons, detection of *S. typhi* and *B. pseudomallei* has now relied on molecular assays such as PCR, which has high sensitivity and specificity, less turn-around time, and is suitable for developing countries. Buffy coat has been shown to be ideal for PCR detection of such intracellular pathogens [16–18, 44]. In our study, culture was performed on whole blood, whereas PCR assays were carried out on DNA extracted from the buffy coat. Because of the monocyte-tropic nature of these two pathogens, buffy coat, which is rich in white blood cells could have yielded positives but not in blood culture.

Conventional diagnosis by blood culture and serological identification using specific antiserum is routinely performed by hospitals and diagnostic laboratories but is less sensitive especially in South and Southeast Asian countries where empirical use of antibiotics is very common and available over the counter. This could be the other reason for *S. typhi* and *B. pseudomallei* negative blood cultures in our study though were positive by multiplex nested PCR. This is a cross-sectional study and follow-up of the patients was not carried out. A blinded clinical study with follow-up is needed to evaluate this test in field conditions.

An automated blood culture system would cost around 17,000 USD and culture for individual pathogens including biochemical identification and confirmatory tests would cost 35 USD in India, as routine and mycobacterial blood cultures are done using separate culture bottles. In contrast, a multiplex nested PCR for the three pathogens would cost only 25 USD. This approach in multiplex format reduces time and cost when compared with culture techniques. In many laboratories, an automated blood culture system is not available, but PCR assays have been introduced for routine screening of blood-borne pathogens in blood donors. Thus, considering the technical issues and trained personnel availability, PCR may be more widely applicable than the blood culture system in developing countries.

5 Conclusion

International travel is increasing and a number of Western travelers develop febrile illnesses during or shortly after their return from the tropics especially from South and Southeast Asian regions. The differential diagnosis is broad and a systematic approach to the management of the febrile patient is required to establish the diagnosis and to initiate prompt and effective antimicrobial treatment. Our study shows that enteric fever, melioidosis, and tuberculosis should be considered separately from other infections such as malaria, dengue, and rickettsial infections in all returning travelers. Detection of *M. tuberculosis*, *S. typhi*, and *B. pseudomallei* by multiplexing can be used for confirming the diagnosis. Thus, the rapid and specific detection of these three organisms in clinical specimens such as the patient's buffy coat samples is very important. Therefore, this study showed the value of a multiplex nested PCR platform for determining the etiologies of PUO, thereby avoiding the misdiagnosis of these pathogens and inappropriate treatment.

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