JMID/
Journal of Microbiology and Infectious Diseases

2017; 7 (1): 21-28
doi: 10.5799/ahinjs.02.2017.01.0248

RESEARCH ARTICLE

Molecular detection of *Burkholderia pseudomallei* in patients with suspected pulmonary and extra pulmonary tuberculosis

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ABSTRACT

Objectives: Since melioidosis mimics tuberculosis clinically and radiologically, there is a need for a rapid diagnostic method to help the clinician to initiate appropriate antimicrobial treatment in order to prevent mortality. Our objective was to standardize a nested PCR for *B. pseudomallei* and its detection in pulmonary and extra pulmonary samples from patients with suspected TB.

Materials and Methods: Archived pulmonary and extra pulmonary samples which were negative for *M. tuberculosis* smear microscopy, culture and PCR were included in the study. DNA was extracted (QiAmp Blood DNA kit, Qiagen, Germany) and conventional nested PCR were carried out to detect the presence of 16S-23S spacer region of B. *pseudomallei*. The DNA was detected by 2% agarose gel electrophoresis and the presence of 251 bp was considered positive.

Results: A total of 55 samples were tested, out of which 9 (16.3%) samples tested positive for *Burkholderia pseudomallei* using nested PCR, which included 5 extra pulmonary and 4 pulmonary samples. These patients belonged to Tamil Nadu 8 (88.8%) and West Bengal 1 (11.1%) both of which are rice growing regions. Among the nine patients who were positive for *B. pseudomallei* by nested PCR, 2 (22%) were receiving empirical anti-tubercular treatment (ATT). Also, these patients encountered co-morbid condition like renal failure, malignancy, diabetes and co-infection with HIV.

Conclusion: We suggest that the patients with symptoms suggestive of both pulmonary and extra pulmonary tuberculosis should be routinely tested for *Burkholderia pseudomallei* by molecular methods for timely initiation of appropriate therapy and avoid unnecessary exposure to ATT. *J Microbiol Infect Dis 2017; 7(1): 21-28*

Keywords: Melioidosis, *Burkholderia pseudomallei*, mimic Tuberculosis, Polymerase chain reaction, rapid detection

INTRODUCTION

Burkholderia pseudomallei is a facultative gram negative organism, opportunistic and intracellular pathogen. It can present with latent disease manifestations similar to Mycobacterium tuberculosis. Melioidosis may occur as a subclinical infection or as localized infection such as abscess, granuloma, pneumonia, meningoencephalitis, sepsis, chronic suppurative infection which can progress to a gram negative septicemia resulting in a multi-organ failure. *B. pseudomallei* thrive well in damp climate and terrain of flooded low-lying plains. Most of the endemic regions include the rice growing regions of South East Asian countries [1,2]. Risk factors like diabetes mellitus, alcoholism, renal disease, chronic liver disease, steroid therapy and malignancy are considered responsible for reactivation and disease progression [3,4]. Routine diagnostic methods include culture of the organism from blood, sputum, pus, urine, synovial fluid, peritoneal fluid, and pericardial fluid. Molecular assays are available only at reference laboratories in endemic countries. Mortality can be prevented by a timely detection and treatment with specific antibacterial agents [5].

METHODS

Patient selection criteria

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Received: 20 February 2016, Accepted: 26 October 2016

A retrospective study was conducted to determine the presence of *B. pseudomallei* by nested PCR. Patients presenting with persistent cough, fever, weight loss, loss of appetite and other signs and symptoms consistent with tuberculosis but negative for AFB smear microscopy, culture & PCR were included in the study. Samples of patients from specialty clinics were considered as low index of suspicion for tuberculosis based on their clinical signs and symptoms consistent with tuberculosis such as fever ,cough, loss of weight, loss of appetite >3 weeks duration [6,7]. Similarly, patients from the RNTCP center were considered as high index of suspicion for tuberculosis [6,7]. Institutional ethics committee approval was obtained (IEC Ref No: IEC-NI/09/DEC/13/39) and informed consent was obtained from patients prior to sample collection. The samples collected were stored at -20° C in multiple aliquots. Clinical details were collected both retrospectively and prospectively.

Nested PCR

A conventional nested PCR for *B. pseudomallei* was done on 36 sputum samples from patients suspected to have tuberculosis and 19 pus samples from patients with suspected extra pulmonary tuberculosis. DNA extraction was performed as per the manufacturer's instructions (Qiagen, Germany). DNA extraction was done using lysis buffer after addition of proteinase-K and incubated for 30 minutes at 56 ^{o}C. The lysed DNA was then precipitated using ethanol and purified with wash buffers. The DNA was eluted and stored at –20 ˚C.

A nested PCR was performed targeting the 16S-23S spacer region [8]. The primer details are listed in Table 1 [8, 9]. The cycling conditions for the first round PCR were 95 °C for 15 min, 30 cycles of 95 °C for 45 s, 52 °C for 40 s, 72 °C for 1 min, and 72 °C for 7 min. The cycling conditions for the second round PCR were 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 60°C for 30 s, 72 °C for 45 s, and 72 °C for 7 min. Two microliter of first product was used as a template for the second round. The second round PCR amplicons were subjected to 2 % agarose gel electrophoresis and were analyzed under Gel documentation unit (BIORAD). The band size of 251 bp was considered positive for 16S-23S spacer region [8]. *B. pseudomallei* plasmid DNA was used as positive control for detection of B. pseudomallei. To avoid PCR carry-over contamination appropriate measures were taken which includes use of negative control (Sterile Milli-Q-water) between every three samples. The study results were checked for inter-reader reproducibility. The test samples which were repeatedly positive were considered positive to insure reproducibility.

Automated Culture

The samples that were positive for the above nested PCR were subjected to automated culture identification using VITEK-2 system [10].

RESULTS

A total of 55 samples were included in this study. Of these, 36 (65.45%) were collected from patients attending specialty clinics and the remaining 19 (34.54%) were collected from patients attending RNTCP center. The overall samples included consist of 19 (34.54%) pus samples and 36 (65.45%) sputum samples; in high index of suspicion group all 19 (34.54%) were sputum samples.

A total of 9 (16%) samples were found to be positive for *B. pseudomallei* (n=55) by nested PCR (Figure 1 and 2), out of which 4 (44%) were sputum samples and 5 (56%) were pus samples. All nine samples tested negative for AFB by microscopy and negative for *M. tuberculosis* by PCR targeting IS6110 and TRC4 region (Figure 3a and 3b). The samples (pus) that were positive by PCR were negative for growth by culture. Analysis of demographic profile of the patients positive for *B. pseudomallei* (n=9) showed mean age to be 45 years and the male / female ratio was 1.25:1. Geographically, most of them belonged to Tamil Nadu which includes 8 (89%) patients but one (11%) to West Bengal. Among the patients who were positive for *B. pseudomallei* by the nested PCR, 7 (78%) attended specialty clinics (low index of suspicion for tuberculosis), out of which 5 (56%) were extra pulmonary samples (pus) and 2 (22%) were pulmonary samples. Similarly, among the patients who were positive for B. pseudomallei, two (22%) attended the RNTCP center (high index of suspicion for tuberculosis). Clinical profiles of the 9 (16%) patients who were positive for *B. pseudomallei* are shown in the table 2 and table 3.

In the 5 (56%) patients who had extra-pulmonary manifestations, the common clinical manifestations observed were site specific pain in 3 (33%) patients, followed by chronic loss of weight in 2 (22%) and persistent fever in one (11%) patient. In this group the major co-morbid conditions encountered were the presence of chronic kidney disease in 2 (22%) patients, malignancy and diabetes mellitus each in one (11%) patient. Also, among patients in low index of suspicion group who were positive for *B. pseudomallei* by nested PCR, 2 (22%) were receiving empirical Anti-tubercular treatment (ATT).

In patients with pulmonary manifestations (n=4), persistent cough and fever were the most common clinical manifestations found among 3 (33%) patients, followed by chronic loss of weight, loss of appetite and/or breathlessness in 2 (22%) patients. They also presented with co-morbid conditions such as chronic kidney disease, malignancy, HIV infection, and alcoholism each in one patient.

Table 1. Primer sequence for the *Burkholderia pseudomallei* PCR targeting 16S-23S spacer region.

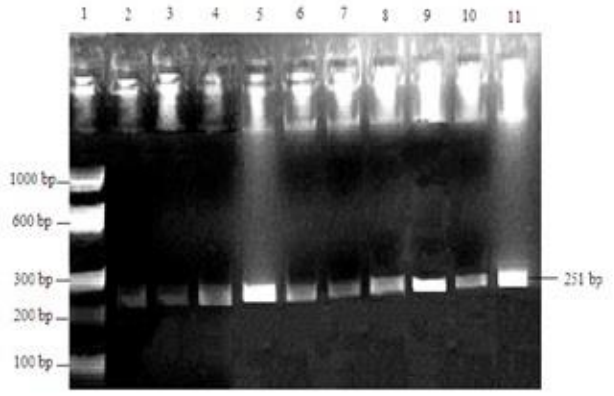
Target gene		Primer sequence (Forward and Reverse)	Base pair Size
16S-23S spacer region	Cycle 1	5' – CGATGATCGTTGGCGCTT – 3' 5' – CGTTGTGCCGTATTCCAAT – 3'	282 bp
	Cycle 2	5'-CCTCCACCAATTGCGATGATCGTT-3' 5'-CAATCACAACCCGGATAGCTTCCAC-3'	251 bp

Table 2. Details of patients positive for *B. pseudomallei* and who presented with extra-pulmonary manifestations (Pus samples).

Sample No	Group	Lab ID	Age, Sex	Pus Site	Smear Positive, Negative	TB-PCR (IS6110, TRC4¬)	*B. pseudomallei* PCR	Co-morbid Conditions	Region
1	Low index of suspicion for TB	1/12	56, Female	Psoas Abscess	Negative	Negative	Positive	Malignancy, CKD	West Bengal
2		2/12	32, Female	Renal Abscess	Negative	Negative	Positive	–	Tamil Nadu
4		4/12	34, Female	Chest wall abscess	Negative	Negative	Positive	–	Tamil Nadu
5		5/13	32, Male	Psoas abscess	Negative	Negative	Positive	CKD	Tamil Nadu
7		7/14	40, Female	Pancreatic abscess	Negative	Negative	Positive	Diabetes mellitus	Tamil Nadu

Table 3. Details of patients positive for B. pseudomallei and who presented with pulmonary manifestations (Sputum samples)

Sample No	Group	Lab ID	Age, Sex	Smear Positive/ Negative	TB-PCR (IS6110, TRC4¬)	*B. pseudomallei* PCR	Co-morbid Conditions	Region
3	Low index of suspicion for TB	3/12	42, Male	Negative	Negative	Positive	Alcoholism	Tamil Nadu
6		6/14	75, Male	Negative	Negative	Positive	Malignancy CKD	Tamil Nadu
8	High index of suspicion for TB	8/12	40, Male	Negative	Negative	Positive	Retrovirus positive	Tamil Nadu
9		9/12	55, Male	Negative	Negative	Positive	–	Tamil Nadu



Lane 1: Molecular Marker 100 bp, Lane 2: 01/2012, Lane 3: 02/2012, Lane 4: 04/2012, Lane 5: 05/ 2013, Lane 6: 07/2014, Lane 7: 03/2012, Lane 8: 06/2014, Lane 9: 08/2012, Lane 10: 09/2012 Lane 11: Positive control at 251 bp

Figure 1. *Burkholderia pseudomallei* as detected by PCR 16S-23S spacer region (251 bp) from clinically suspected TB.

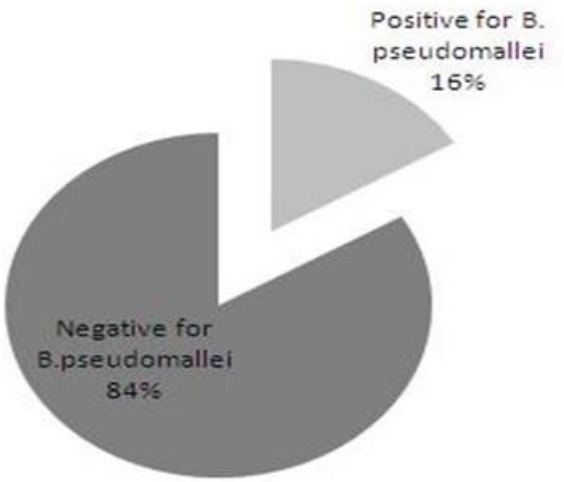
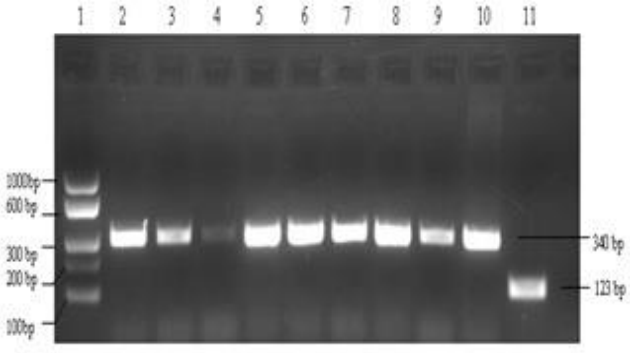
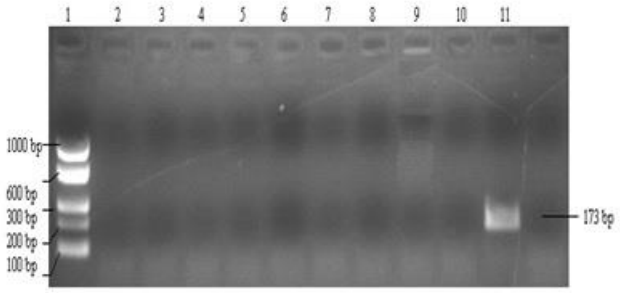


Figure 2. Proportion of cases of Melioidosis among patients with suspected tuberculosis.



Lane 1: Molecular Marker 100 bp, Lane 2: 01/2012, Lane 3: 02/2012, Lane 4: 04/2012, Lane 5: 05/ 2013, Lane 6: 07/2014, Lane 7: 03/2012, Lane 8: 06/2014, Lane 9: 08/2012, Lane 10: 09/2012 Lane 11: Positive control at 123 bp

Figure 3 (a). PCR targeting IS6110 specific to *M. tuberculosis*.



Lane 1: Molecular Marker 100 bp, Lane 2: 01/2012, Lane 3: 02/2012, Lane 4: 04/2012, Lane 5: 05/ 2013, Lane 6: 07/2014, Lane 7: 03/2012, Lane 8: 06/2014, Lane 9: 08/2012, Lane 10: 09/2012 Lane 11: Positive control at 173 bp.
Figure 3 (b). PCR targeting *TRC4* specific to *M. tuberculosis*.

DISCUSSION

Melioidosis is a potentially fatal infectious disease endemic in Southeast Asia; it has been reported to be under diagnosed in India [11]. After the 2004 Southeast Asian tsunami, an increase in the number of melioidosis cases was observed in these areas [8]. Also, the incidence of melioidosis in tropical countries has generally increased as there is an increase in immune compromised especially people with diabetes mellitus and renal disease [1,12]. In our study, co-morbid conditions encountered include chronic kidney disease (n=3), malignancy (n=2), diabetes mellitus (n=1), HIV infection (n=1) and alcoholism (n=1). These co-morbidities have been previously reported in melioidosis [1,12].

Pulmonary condition like pneumonia is a common manifestation of *B. pseudomallei*. Subacute pneumonia or chronic pneumonia with low grade fever may be mistaken for pulmonary tuberculosis. Melioidosis can also present as an organ specific or localized disease such as intra-abdominal abscess [13,14]. We found that out of the five pus samples positive for B. pseudomallei, 3(33%) were from patients who had deep seated abscess of which two were in the psoas region and one was a renal abscess, the rest included one patient with a pancreatic abscess and a chest wall abscess.

In our study, *B. pseudomallei* was detected in 9 (16%) samples from patients suspected to be having pulmonary tuberculosis as well as extra pulmonary tuberculosis. These nine samples tested negative for smear microscopy and PCR for *M. tuberculosis* targeting IS6110 & TRC4 region. Further, 19(34.54%) samples from the high index of suspicion group were also confirmed to be negative for *M. tuberculosis* by culture method (Lowenstein Jensen (LJ) media). To detect *M. tuberculosis* a combination of laboratory methods such as microscopy, culture are required. Smear microscopy is rapid and easy to perform but has been reported to have lower sensitivity when compared to culture. Culture method though considered as gold standard, they are time consuming and requires sophisticated laboratories [15,16]. Often in TB endemic areas, empirical ATT may be started in the absence of any laboratory evidence.

Among the PCR positives, we have subjected the 5 pus samples to culture using VITEK-2 system. None of these samples showed growth on culture. Since all the patients had prior exposure to antibiotics for persistent fever, this could account for 'no growth in culture' [8,17]. We have documented culture negativity in pus samples. This has been reported in another study from South India, where blood culture was reported negative due to low bacterial load [8,9]. It has also been reported that often the identity of *B. pseudomallei* is by PCR method and the reason could be due to increased accuracy of the nested PCR targeting the 16S-23S rRNA spacer region (limit of detection: 1 colony forming unit/ 5µL PCR reaction for *B. pseudomallei*) [8,9]. In the case of sputum samples, we did not have enough material to do culture. This is because, earlier we did multiple tests with the sputum samples such as AFB smear microscopy, culture (using LJ medium) and PCR for TB targeting IS6110 and TRC4 region (a part of another study).

Identification of *B. pseudomallei* by culture using selective medium such as Ashdown's medium, requires minimum of 48-72 hours for identifica-

tion. Since *B. pseudomallei* is a non-fermentor, growth on non-selective medium will take at least a week to biochemically and phenotypically confirm the diagnosis, moreover handling of *B. pseudomallei* cultures requires special conditions such as Biosafety level 3. Therefore, PCR targeting specific region can be used for early detection of B. pseudomallei. Different primers have been used for rapid detection of *B. pseudomallei* such as 23S rRNA gene, mpr gene, fur gene [18]. PCR targeting 16S spacer region used for detection of *B. pseudomallei* has been reported with increased sensitivity [8,9]. Molecular methods using nucleic acid amplification techniques by PCR are rapid method for identification of *B. pseudomallei* and can be done directly on clinical samples. Earlier studies have documented the use of a conventional uniplex PCR targeting 16S-23S rRNA spacer region for *B. pseudomallei* in blood samples. Subsequently the uniplex PCR was developed into a nested PCR which had improved the sensitivity [8,9,12,18]. Molecular methods for diagnosis of B pseudomallei not only help in rapid diagnosis and treatment initiation, but can also reduce the risk in handling of the cultures.

Contamination in PCR is likely to remain problematic. However measures to ensure quality control and quality assurance are routinely followed in the laboratory [19]. We have carried out the work in a dedicated PCR laboratory where culture or multiple assays are not done. As a good laboratory practice, we maintain use of three room set-up, dedicated instruments and sterile consumables [19,20]. Reagents were handled inside laminar flow hood, where we prepare and aliquot reagent mix into PCR tubes. Later, these tubes were opened only once to add DNA inside a bio-safety cabinet. These work areas are cleaned with DNase away and subjected to UV sterilization as per standard operating procedure (SOP). Gloves were changed between additions of each DNA and disposable sterile tips with filter (barrier) were used to ensure no sample to sample contamination. Consumables, instruments and protective clothing used in the processing area were not taken back into the reagent preparation area. Thus, the unidirectional work flow was maintained. To ensure no amplification error, we have included positive control and followed optimal cycling conditions for each run. To identify any cross-contamination or carry-over contamination, we have included negative controls between every three samples and reagent control or no template control to identify reagent contamination if any [19,20]. We believe in the integrity of the PCR test result since these quality measures have been strictly followed. In our study, to ensure the reliability of the PCR positive results the positive samples were repeatedly tested by two persons (EJ & RB). We therefore believe that there was no scope for false positivity due to carry-over contamination or cross-contamination in our results. Previous reports suggest that the primers targeting 16S-23S spacer region to be highly sensitive and specific as it did not amplify or cross-react with other heterologous targets [8,9].

We performed PCR on samples obtained from patients with specific signs and symptoms of tuberculosis such as persistent cough and fever, chronic loss of weight, loss of appetite, breathlessness and site specific pain (Table 2 & 3). Patients who present with fever for prolonged duration are usually treated as TB, because TB is endemic; *B. pseudomallei* may not be looked for. We have identified *B. pseudomallei* DNA in 9 patients (five pus and four sputum samples) who were clinically suggestive of melioidosis [21-23]. To prevent misdiagnosis and for initiation of appropriate therapy as well as avoiding unnecessary exposure to anti-tubercular drugs we suggest to carryout screening for *B. pseudomallei* by PCR tests in patients who have signs and symptoms suggestive of tuberculosis.

Demographic analysis of all nine patients diagnosed as melioidosis has shown that all patients belong either to Tamil Nadu or West Bengal, states known for the cultivation of rice (Table 2 & 3). We also found in our study that out of the nine patients who were positive for *B. pseudomallei* by nested PCR, two patients presented to RNTCP center as they had symptoms highly suggestive of tuberculosis and the remaining seven patients presented to different specialties clinics like Nephrology, Orthopedic, Obstetrics and Gynecology, General Surgery and General medicine suggesting the varied clinical presentation of melioidosis. To our knowledge this is the first study to document the presence of *B. pseudomallei* in patients with low and high index for suspicion of tuberculosis. We suggest that in

melioidosis endemic areas, *B. pseudomallei* be ruled out in patients presenting to such clinics.

Acknowledgements: We thank Dr. Sathish Sankar, Associate Professor / Senior Scientist, Sri Narayani Hospital and Research Centre, Sripuram, Vellore 632 055, Tamil Nadu, India for kindly providing us the positive control for *B. pseudomallei.*

Declaration of conflicting interests: The authors declared no conflicts of interest with respect to the authorship and/or authorship of this article.

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