A diagnosis of *Burkholderia pseudomallei* directly in a bronchoalveolar lavage by polymerase chain reaction

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**Abstract**

Melioidosis is an infectious disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*. Interest in the molecular identification of *B. pseudomallei* has increased after its classification as a category B agent by the US Centers for Disease Control and Prevention. The present article reports a diagnosis of *B. pseudomallei* directly in a bronchoalveolar lavage by polymerase chain reaction amplification. The results obtained show that direct detection of the 16-23s spacer sequence in bronchoalveolar lavage is a quick and specific test to diagnose melioidosis.

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Melioidosis is a potentially fatal infection caused by the facultative intracellular bacterium *Burkholderia pseudomallei* (Merritt et al., 2006). Melioidosis produces diverse clinical manifestations, including acute septicemia, pulmonary infection, and chronic visceral and soft tissue abscesses (Supaprom et al., 2007). The clinical symptoms of melioidosis mimic those of many other diseases. Therefore, differentiating between melioidosis and other acute and chronic bacterial infections is often difficult (Peacock et al., 2008). Definitive identification of *B. pseudomallei* relies on an extensive set of biochemical tests that may require up to 5 days before results are obtained.

Practical difficulties for the diagnostic laboratory include the presence of closely related *Burkholderia* spp. in specimens from nonsterile sites and atypical colony morphology of some *B. pseudomallei* strains (Lee et al., 2007). Also, handling of *B. pseudomallei* cultures is a high-risk activity and is limited to biosafety level 3 laboratories (Peacock et al., 2008). Consequently, the molecular identification may offer a rapid alternative, especially in first-line laboratories equipped to perform DNA amplification methods such as diagnostic polymerase chain reaction (PCR) (Merritt et al., 2005). Various PCR procedures for *B. pseudomallei* have been developed, but most of them have only been evaluated using pure bacterial cultures (Supaprom et al., 2007). Recently, real-time PCR assays reported the rapid detection of *B. pseudomallei* through DNA extraction of clinical blood samples (Meumann et al., 2006; Novak et al., 2006; Supaprom et al., 2007). However, although this methodology is rapid and specific for detection of *B. pseudomallei*, it is not applicable for routine diagnostic purposes in many laboratories.
The present article reports a diagnosis of *B. pseudomallei* directly in a bronchoalveolar lavage by PCR amplification of the specific 16-23s spacer region.

In April 2008, a case of melioidosis in a 17-year-old boy was reported to the Ceará State Health Secretariat. The patient probably contracted the disease in Ubajara (3°51'16" S, 40°55'16" W), Ceará, in northeastern Brazil, while swimming in a recreational river/waterfall in that region. The bronchoalveolar lavage obtained from this patient was sent to the Specialized Medical Mycology Center (CEMM) to investigate the causative agent of the disease. This was done by PCR assay directly from bronchoalveolar lavage and, concomitantly, by culture in blood agar, *B. pseudomallei* selective agar, and Ashdown agar. The culture was identified phenotypically according to the criteria described for Gram-negative nonfermentative bacilli and by characteristics such as motility and bipolar stain (Virginio et al., 2006). The colonies that indicate the possibility of *B. pseudomallei* were submitted to the oxidase test and colistin (10 μg/mL) sensitivity test through disk diffusion in Müller–Hinton medium. The bacterium was resistant to colistin and was oxidase positive. Then, the *B. pseudomallei* diagnosis was confirmed through API20NE (BioMerieux, Marcy l’Etoile, France) (Inglis et al., 2004).

For molecular diagnosis, the total genomic DNA from 2 mL of bronchoalveolar lavage was extracted and purified using a protocol for cell culture from the WIZARD® Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer’s instructions. The concentration and purity of the DNA preparation (170 ng/μL) were determined by measuring the optical density at 260 and 280 nm using a spectrophotometer (Ultrospec 1100 pro; Amersham Bioscience, Piscataway, NJ). PCR was performed according to Merritt et al. (2006) with minor modifications. The reaction was performed in a final volume of 25 μL containing 10 μL of DNA sample (30 ng/μL), 2.5 μL of buffer (New England Biolabs, UK), 1 mmol/L of MgCl₂ (Invitrogen, Carlsbad, CA), 50 pmol each of the primers Bp1 (5′-CGATGATCGTTGGGCTT-3′) and Bp4 (5′-CGTTGTGCCTTACCAAT-3′), 10 mmol/L of pooled deoxynucleoside triphosphates, and 1 U of Taq polymerase (New England Biolabs). PCR comprised 4 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C, with a final hold for 7 min at 72 °C. PCR products were demonstrated by ethidium bromide gel electrophoresis on 1% agar gel and visualized under ultraviolet light.

A control *B. pseudomallei* strain (CEMM BP 01) used in this study was isolated in northeastern Brazil in 2003 (Rolim et al., 2003) and belongs to the CEMM collection. The control *B. pseudomallei* strain was initially cultured on blood agar medium at 37 °C for 24 to 48 h. A single colony was then inoculated into 10 mL of brain–heart infusion broth and incubated at 37 °C overnight. For the extraction of genomic DNA, the protocol for Gram-negative bacteria of the WIZARD® Genomic DNA purification kit was used, according to the manufacturer’s instructions. Aliquots of DNA obtained from the control strain and from bronchoalveolar lavage after first treatment with buffer lysis were incubated in brain–heart infusion broth at 37 °C for 7 days. The absence of any growth assured the safety of the procedure. Determination of the concentration and purity of the DNA (407.5 ng/μL) and the PCR assay of the control strain were performed as described for bronchoalveolar lavage specimens.

PCR yielded amplification showing products of approximately 300 bp from both bronchoalveolar lavage and the control strain (Fig. 1). Negative control using molecular biology grade water instead of DNA preparations and a bronchoalveolar lavage of 2 patients without suspected for melioidosis did not yield any amplification products.

The present study using PCR assay to detect a specific DNA sequence directly in bronchoalveolar lavage is very interesting because pulmonary melioidosis is the most well-known clinical presentation of the disease (Currie, 2003). Thus, this specific PCR amplification may be a rapid and sensitive method for diagnosis of melioidosis.

The PCR amplification of DNA from both the bronchoalveolar lavage and control strain showed a single amplification product of 302 bp. Merritt et al. (2006) identified correctly 71 of 72 *B. pseudomallei* isolates by PCR analysis, which is able to differentiate *B. pseudomallei* from other bacteria, including *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia thailandensis*, *Burkholderia viet-namiensis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Mycobacterium tuberculosis*.

DNA–DNA homology and cellular lipid and fatty acid composition revealed that *B. pseudomallei* and Burkhol-

![Fig. 1. Agarose gel electrophoresis of PCR products produced from *B. pseudomallei*. Lane 1, 100-bp ladder marker; 2, bronchoalveolar lavage sample; 3, control culture (strain number CEMM BP 01). The 302-bp PCR amplification product specific for *B. pseudomallei* is indicated.](image-url)
B. mallei are very closely related to each other. The high homology between the 2 species in DNA sequences is shown in genes, such as flagellin and 16S ribosomal RNA, thus, making it difficult to differentiate them by molecular identification systems (Lee et al., 2005).

Although B. pseudomallei and B. mallei are very closely related, the importance of this PCR protocol is not impaired, because they differ considerably in other aspects, including ecologic behavior. For instance, B. pseudomallei is to a large extent a free-living organism, readily isolated from soil and surface water in endemic areas and only on rare occasions finds a niche in animal hosts. B. mallei, however, behaves more successfully with little host specificity and is considered to be an obligate parasite by some investigators. One main morphologic distinction between these 2 species is the presence of flagella in B. pseudomallei and their absence in B. mallei (Anuntagool and Stitaya, 2002).

Melioidosis has been considered an emerging disease in Brazil since the first cases reported in the northeast region (Rolim et al., 2005). Thus, the recent detection of melioidosis in northeastern Brazil highlights the extent of its distribution in the Americas and underlines the need for improved diagnostic methods (Inglis et al., 2006). Currently, the greater reliability, shorter time for results, simplicity, and lower cost of conventional PCR make it the leading method to confirm B. pseudomallei diagnosis.

The present study used an alternative method for safe DNA extraction from bronchoalveolar lavage, and this protocol may reduce the time required to obtain definitive diagnostic results, respecting the presumptive diagnosis of melioidosis, which correctly identified B. pseudomallei even before the confirmation by culturing, which occurred after 5 days. Also, it is easily applicable for routine diagnostic purposes in many laboratories in developing countries, which have our reality.

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References


