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Development of a polymerase chain reaction assay for the specific identification of *Burkholderia mallei* and differentiation from *Burkholderia pseudomallei* and other closely related Burkholderiaceae☆

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Abstract

Burkholderia mallei and *Burkholderia pseudomallei*, the etiologic agents responsible for glanders and melioidosis, respectively, are genetically and phenotypically similar and are category B biothreat agents. We used an in silico approach to compare the *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 genomes to identify nucleotide sequences unique to *B. mallei*. Five distinct *B. mallei* DNA sequences and/or genes were identified and evaluated for polymerase chain reaction (PCR) assay development. Genomic DNAs from a collection of 31 *B. mallei* and 34 *B. pseudomallei* isolates, obtained from various geographic, clinical, and environmental sources over a 70-year period, were tested with PCR primers targeted for each of the *B. mallei* ATCC 23344-specific nucleotide sequences. Of the 5 chromosomal targets analyzed, only PCR primers designed to $bimA_{Bm}$ were specific for *B. mallei*. These primers were used to develop a rapid PCR assay for the definitive identification of *B. mallei* and differentiation from all other bacteria.

Keywords: *Burkholderia*; Glanders; Melioidosis; PCR

1. Introduction

Both *Burkholderia mallei* and *Burkholderia pseudomallei* are closely related Gram-negative bacterial pathogens that have the capability to cause severe human and animal disease. *B. mallei*, the causative agent of glanders disease, is a host-adapted equine pathogen that has been shown to be a clone of *B. pseudomallei* (Godoy et al., 2003; Waag and DeShazer, 2004). *B. mallei* primarily infects horses, donkeys, and mules, whereas humans are considered an incidental host. With the development of motorized transportation in the early 20th century and implementation of quarantine precautions for imported animals, no naturally occurring human cases of glanders have been reported in the United States since the 1930s (Waag and DeShazer, 2004). However, there are sporadic incidences that still occur in Asia, the Middle East, South America, and Africa. Human glanders occurs in individuals such as veterinarians, slaughterhouse workers, and laboratory scientists whose occupation exposes them to the pathogen. In solipeds, 2 distinctive forms of glanders may arise: acute (observed in mules and donkeys) and chronic (common in horses). Like solipeds, both acute and chronic forms of glanders can exist in humans, depending on the route of exposure (i.e., aerosol versus cutaneous). Human acute

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glanders is characterized by fever and fatigue as well as inflammation of and nodule formation on the face and peripheral limbs (Neubauer et al., 1997). Chronic glanders in humans presents with swollen lymph nodes, ulcerating nodules in the alimentary and respiratory tracts, weight loss, and numerous subcutaneous abscesses. Human glanders is a lethal disease that is essentially indistinguishable from human melioidosis (caused by *B. pseudomallei*), and if appropriate antibiotics are not administered, death normally occurs (Neubauer et al., 1997; Waag and DeShazer, 2004). Melioidosis, caused by the Gram-negative bacterium *B. pseudomallei*, is endemic in tropical regions throughout the world with the most prevalent regions being Southeast Asia and northern Australia (Cheng and Currie, 2005). Sporadic cases of melioidosis have been reported in the Indian subcontinent, Central and South America, the Caribbean, Africa, Iran, the Pacific Islands, and France in the early 1970s (Dance, 2002). *B. pseudomallei* is a facultative intracellular bacterium that is considered to be an opportunistic pathogen for humans, in particular, individuals with underlying risk factors including diabetes mellitus, alcoholism, and renal complications (Woods et al., 1999). *B. pseudomallei* can be acquired from contaminated environmental samples through inhalational or cutaneous routes of infection (Cheng and Currie, 2005). Symptoms of melioidosis are much like those of glanders and may include acute or chronic pneumonia, acute septicemia, and latent infections that can persist for years.

Because of the potential for *B. mallei* and *B. pseudomallei* weaponization, the rapid and definitive identification of these highly infectious pathogens is essential for the immediate initiation of appropriate antibiotic therapy. The current methods for diagnosing *B. mallei* and *B. pseudomallei* incorporate various biochemical and substrate utilization tests, cellular and colony morphology analysis, and motility assays, which can take up to 1 week to complete (Cheng and Currie, 2005; Neubauer et al., 1997). Furthermore, relying on biochemical testing alone for the identification of *B. mallei* and *B. pseudomallei* using readily available commercial kits (API 20NE and RapID NF) has recently been shown to be 0–60% accurate, at best (Glass and Popovic, 2005). These commercial systems may also falsely identify other organisms as *B. pseudomallei* and *B. mallei*.

Considering the high level of genetic, biochemical, and phenotypic similarities between *B. mallei*, *B. pseudomallei*, and *Burkholderia thailandensis* (Brett et al., 1997; Godoy et al., 2003; Holden et al., 2004; Nierman et al., 2004), molecular and biochemical approaches for identifying and differentiating these closely related *Burkholderia* species are problematic. There have been several reports in the literature that have targeted the *B. pseudomallei* 16S and 23S rRNA, 16S-23S intergenic region, flagellin C (*fliC*), heat shock protein 70, and a type 3 secretion (TTS) system for the molecular identification of *B. pseudomallei* and possible differentiation from *B. mallei* (Antonov et al., 2004; Gee et al., 2003; Hagen et al., 2002; Lee et al., 2005; Sprague et al., 2002; Tanpiboonsak et al., 2004; Thibault et al., 2004; Tomaso et al., 2005; Tomaso et al., 2004; Tyler et al., 1995). Despite the numerous misleading titles in the literature (i.e., titles that imply *B. mallei*-specific), there are no *B. mallei*-specific molecular assays for discriminating this obligate mammalian pathogen from *B. pseudomallei*.

The recent availability of complete genome sequences for *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 (Holden et al., 2004; Nierman et al., 2004) has greatly facilitated the ability to identify unique *B. mallei* DNA sequences. Using comparative in silico DNA sequence analysis, we identified a unique nucleotide sequence that is highly conserved among all virulent *B. mallei* isolates. From these data, a *B. mallei*-specific polymerase chain reaction (PCR) assay was developed and tested for specificity against a panel of diverse clinical and environmental isolates of *B. pseudomallei* and other closely related Gram-negative species.

2. Materials and methods

2.1. Bacterial strains used in this study

An extensive representative panel of 31 *B. mallei*, 34 *B. pseudomallei*, and 12 *B. thailandensis* strains isolated from various geographic, clinical, and environmental locations throughout the world were investigated (Table 1). Additional *Burkholderia* species tested in this study include *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia multivorans*, and *Burkholderia vietnamiensis*. Other Gram-negative and Gram-positive isolates analyzed for cross-reactivity with the *B. mallei*-specific assay include *Escherichia coli*, *Chromobacterium violaceum*, *Yersinia pestis*, *Pseudomonas aeruginosa*, and *Bacillus anthracis*. Genomic DNA for PCR amplification was purified using previously described methods (Wilson, 1987), and template DNA for *Bacillus anthracis* was kindly provided by Dr Donald Chabot, United States Army Medical Research Institute of Infectious Diseases. All *B. mallei* isolates were cultured using Luria–Bertani (LB) broth containing 4% glycerol (LBG) (Sigma, St. Louis, MO). With the exception of *Y. pestis*, which was grown on sheep blood agar plates (agar plate scrapings were used for genomic DNA purification), all other strains used in this work were grown in LB broth. *C. violaceum* and *Y. pestis* were propagated at 25 °C, whereas the remaining bacterial isolates were cultured at 37 °C with aeration.

2.2. In silico genomic subtraction

Using a combination of Critica and Glimmer, total open reading frames (ORFs) of *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 were overpredicted from 2003 draft versions of their genomic sequences. The approximately 6200 *B. mallei* ORFs were compared to those of *B. pseudomallei* using BLASTP (Ver. 2.1; web interface: low complexity filter, off; E-value cutoff, 100; -b, 1; -v, 1).