International Journal of Medical Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Evaluation of *fimC* and *bdha* based duplex PCR for specific identification and differentiation of *Burkholderia pseudomallei* from near-neighbor *Burkholderia* species

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ARTICLE INFO

Keywords:
bdha
fimC
IAC
Burkholderia duplex PCR
Burkholderia pseudomallei

ABSTRACT

Assays for the rapid detection and accurate differentiation of *Burkholderia pseudomallei* from near-neighbor species are urgently needed in melioidosis endemic regions due to the high associated mortality and biowarfare importance of the pathogen. PCR-based methods have revolutionized this field due to the accuracy, sensitivity, and specificity that are achievable in a rapid way. In this study, a compound molecular detection system, consisting of a duplex PCR assay, was developed for the specific identification of *Burkholderia pseudomallei* and differentiation from other *Burkholderia* species. For accurate identification of *B. pseudomallei*, we deciphered and adopted a novel gene termed putative fimbrial chaperone (*fimC*). D-beta hydroxybutyrate dehydrogenase (*bdha*), reported previously by our group for sequence-based differentiation of *B. pseudomallei* from other *Burkholderia* species, was employed as a genus-specific target. Enforcement of an internal amplification control in the PCR format ruled out possible false negative results in the assay. Thus, the developed PCR assay was highly specific (100%) in its detection features, and a clear detection sensitivity of 10 pg/μl for purified gDNA and 3×10^3 CFU/ml for *B. pseudomallei* spiked urine was recorded. Successful identification of *B. pseudomallei* from an experimental mouse model at both the genus and species level revealed the accurate diagnostic efficiency of the duplex PCR method.

1. Introduction

Members of the genus *Burkholderia* include bacteria of multi-host pathogens related to emerging infectious diseases as well as biotechnological importance. In recent decades, the genus has gained global attention due to two species, namely, *Burkholderia pseudomallei* and *Burkholderia mallei*, which are recognized as Category B biothreat agents by the Centre for Drug Control (Rotz et al., 2002). *Burkholderia pseudomallei* causes melioidosis in humans, and *Burkholderia mallei* causes glanders in horses, mules and donkeys (Lowe et al., 2014). The high mortality rates of melioidosis and glanders, aerosolization and low infectious dose make the causative agents usable as biological weapons (Lowe et al., 2014). Glanders was eradicated from domestic animals in the West, and very few cases are being encountered in countries of Southeast Asia and parts of Africa (Lee et al., 2005).

Burkholderia pseudomallei has remarkable ability to persist in diverse ecological niches and expand in global distribution, and it has emerged as an opportunistic pathogen causing fatal melioidosis and infection in cystic fibrosis patients (O'Carroll et al., 2003). The recommended gold standard method of identification is isolation, which takes no less than 2–7 days (Gregory and Waag, 2007). This delay in identification entails a delay in therapy, resulting in high mortality (Lowe et al., 2014). As an additional challenge, this pathogen possesses intrinsic resistance to antibiotics (LiPuma, 1998; Gilad, 2007), making therapeutic management difficult. Diagnostic laboratories have found identifying *B. pseudomallei* based on colony morphology and substrate utilization panels such as API and Vitek systems to be unreliable (Inglis et al., 1998; Glass and Popovic, 2005). A major hurdle faced by the scientific community in this context is the high degree of phenotypic and genotypic similarity presented by the members of *Burkholderia* (Lowe et al., 2016). *B. pseudomallei* is a clonal descendant of *B. mallei* that is often found to be difficult to differentiate (Holden et al., 2014). With continuous expansion of the genus, identifying *Burkholderia pseudomallei* and differentiating it from near-neighbor species is also consistently becoming difficult (Ginther et al., 2015).

In India, cases of melioidosis are increasing, and the pathogen is

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https://doi.org/10.1016/j.ijmm.2017.11.007
Received 11 August 2017; Received in revised form 18 November 2017; Accepted 19 November 2017

considered to be endemic (Gopalakrishnan et al., 2013). In our recent study, we reported the prevalence of *Burkholderia pseudomallei* along with other *Burkholderia* species in the South Indian coastal region, which indicated the possibility of exposure of habitats to this infectious bacterium (Peddayelachagiri et al., 2016). The isolation and confirmation of *B. pseudomallei* routinely necessitates pure culture and involves exposure risk to laboratory workers. This problem can be surmounted by adopting rapid and accurate molecular detection methods. PCR-based methods have revolutionized the development of rapid, sensitive and specific detection of *Burkholderia* species. For instance, *recA* based PCR methods have been successfully used as a routine scheme for identifying *Burkholderia cepacia* complex members, which include opportunistic pathogens in cystic fibrosis patients (Vermis et al., 2002; Drevinek and Mahenthiralingam, 2010; Payne et al., 2005). In an attempt to successfully differentiate *Burkholderia pseudomallei* from near-neighbor species, our group developed a novel PCR followed by a sequencing method employing a highly conserved gene, D-beta hydroxybutyrate dehydrogenase (*bdha*) (Peddayelachagiri et al., 2016). The reported *bdha* PCR assay was accurate for identification of *Burkholderia* genus, and subsequent sequence analysis of the species initiated specific species identification. Since sequencing causes a delay in identification and requires highly skilled personnel to evaluate each sample, we intended to further fortify the detection method by including a species-specific gene target in the PCR assay, so that downstream sequencing and analysis could be avoided for ease of performance and identification.

With this background, in the present study, we developed a compound detection system involving a novel duplex PCR assay for identification of *Burkholderia* species to meet the need of the hour. The PCR assay was designed to efficaciously identify the complete *Burkholderia* genus and differentiate *B. pseudomallei* from the rest of the species. The format was developed targeting D-beta hydroxybutyrate dehydrogenase (*bdha*) and putative fimbrial chaperone (*fimC*), where *bdha* serves as a *Burkholderia* genus-specific target and *fimC* as a *B. pseudomallei*-specific target. The novel gene *fimC* was characterized by in silico analyses for reliability in specific detection of *B. pseudomallei.* The sensitivity and specificity of the duplex PCR was also evaluated for its ability to identify *Burkholderia* species at genus level and *B. pseudomallei* at a species level. A simultaneous study was carried out to examine the possibility of deploying the PCR assay as a diagnostic tool by trialing it over a mouse infection model, as well as by spiking a human urine sample with *B. pseudomallei*.

2. Materials and methods

2.1. Ethical statement

Four-week-old female BALB/c mice were obtained from Central Animal Facility, Defence Food Research Laboratory and housed under pathogen-free conditions. The animal experiment was performed with the approval and written consent of the Animal Welfare and Research Ethics Committee of the Defence Food Research Laboratory.

2.2. Bacterial strains

The bacterial strains used in this study are listed in Table 1. The strains included standard reference strains, clinical isolates and environmental isolates of major clinically important *Burkholderia* species and non-*Burkholderia* strains with close phylogenetic relatedness to *Burkholderia* genus. The reference strains were procured from American Type Culture Collection (ATCC), USA, National Collection of Type Cultures (NCTC), UK, Microbial Type Culture Collection (MTCC) and National Collection of Industrial Microorganisms (NCIM) India. The clinical and environmental isolates of *Burkholderia* were confirmed by conventional biochemical tests, MALDI-TOF (Bruker Daltonik Microflex MALDI-TOF mass spectrometer (Bremen, Germany), MALDI Biotyper flex Analysis software version 2.0), and sequencing of *recA* and 16S rRNA. *Burkholderia pseudomallei* NCTC 10724 was employed for optimization of the duplex PCR assay. Standard strains and isolates of other bacteria closely related to *B. pseudomallei* were used for specificity evaluation of the duplex PCR assay. All bacterial strains were cryopreserved in glycerol at −80 °C and cultured in trypticase soy broth (TSB) and brain heart infusion (BHI) broth (Himedia, India) when required.

2.3. Specific targets and primers for detection of genus Burkholderia and species B. pseudomallei

The whole genome sequence of *B. pseudomallei* strain K96243 (accession no. BX571966) available on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) was employed to determine specific target genes. For a genus-specific target, the *bdha* gene (BPSS0017) from our previous study (Peddayelachagiri et al., 2016) was selected. Putative fimbrial chaperone (*fimC*) (BPSS0121) was selected as a species-specific gene target. In Basic Local Alignment Search Tool (BLASTN) analysis, the genes were analyzed for the unique detection specificity required for the study. Conservation and variation of *fimC* within *Burkholderia pseudomallei* strains were analyzed in silico using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), as described earlier (Peddayelachagiri et al., 2016). Table 2 lists the primers used in the present study with the respective oligonucleotide sequence, target gene and amplicon size. For the genus-specific *bdha* target, primers *bdha*-F and *bdha*-R reported previously were adopted (Peddayelachagiri et al., 2016). Care was taken to select primers for *fimC* amplification in highly conserved regions of the gene as determined in Entropy plot.

2.4. Monoplex PCR optimization

Total genomic DNA (gDNA) was isolated from bacterial strains used in this study according to a protocol described elsewhere (Sambrook et al., 1989). Each prepared DNA sample was spectrophotometrically quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific Nanodrop, USA) and stored in aliquots frozen at −20 °C until use. For initial standardization of the detection system, monoplex PCR was performed using the primer sets and respective gDNA as template. Each 25-µl PCR reaction contained 1 U of *Taq* polymerase (Sigma, India), 50 µM of each deoxynucleotide triphosphate (Fermentas, India), 1 x PCR buffer, 2.0 mM of $MgCl_2$, 0.6 pmol of each oligonucleotide primer, and 20 ng of template DNA. Thermal cycling was carried out in a Master Cycler-Pro thermal cycler (Eppendorf, Germany) with an initial denaturation for 4 min at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension at 72 °C for 45 s, with a final 8 min extension at 72 °C. Approximately 10 µl of each PCR product was visualized by agarose gel (2% w/v) electrophoresis.

2.5. Internal amplification control

Based on the guidelines of the European Standardization Committee and International Standard Organization (Hoorfar et al., 2004a, 2004b), an internal amplification control (IAC) was standardized in the present study to prevent false negative results in PCR. pUC19 plasmid sequence available on the NCBI database (accession no. L09137) was used to develop the IAC. A primer pair (Table 2) was designed with 3′ ends complementary to 830 bp of the pUC19 plasmid and 5′ ends with overhangs identical to the primer sequence of *bdha* yielding an 869 bp PCR amplicon inclusive of 5′ flanking region. BLASTN program was used to evaluate the specificity and authenticity of the designed IAC primer pair. The 869 bp PCR amplicon was purified, subjected to 1000-fold dilutions from 1:1000 to 1:10000; PCR amplified simultaneously with the *bdha* target sequence in a single PCR reaction, using a *bdha*