Introduction

The bacterial species *Burkholderia pseudomallei* and *B. mallei*, though genetically very similar, have divergent lifestyles. *B. pseudomallei* is a soil saprophyte and facultative pathogen and the cause of melioidosis, while *B. mallei* is an obligate pathogen and the cause of glanders. Melioidosis is mostly a disease of humans and animals in Southeast Asia and Northern Australia, while *B. pseudomallei* is present in the environment; infection mainly results from percutaneous inoculation or inhalation or aspiration of the organism. Clinical manifestations of melioidosis can be asymptomatic, localized to virtually any organ, or disseminated, though the primary presentations are pneumonia and sepsis, where mortality rates are significant [1,2]. Glanders is mainly an equine disease found in much of the world, except for North America, Europe and Australia, with transmission to humans occurring primarily through direct contact with animals and aerosols [3,4]. Clinical manifestations of glanders in humans are similar to those of melioidosis [1,5]. Both species of bacteria cause fast-progressing diseases and both are intrinsically resistant to several antibiotics. As such, the rapid detection and identification of these species is essential for immediate appropriate patient therapy. Both species are also potential bioterrorism agents, deemed by the U.S. Centers for Disease Control and Prevention Category B Select Agents [6], and simultaneous differentiation when necessary. Alternative diagnostic methods to identify *B. pseudomallei* and *B. mallei* now exist that utilize various platforms: Sanger sequencing [11], multiplex PCR [12], real-time PCR [13,14,15,16], and isothermal DNA amplification [17]. Several of these assays show promise as rapid alternatives to biochemical tests; however few have been extensively validated for robustness and specificity. *B. pseudomallei* and *B. mallei* are relatively genetically promiscuous, making development of robust, specific single-locus assay diagnostics challenging [18,19].

A single-reaction real-time PCR Taqman allelic discrimination assay was previously developed to identify and differentiate *B. pseudomallei* and *B. mallei* [20]. Further analysis of this assay against larger strain collections revealed some false positive identification: a strain of *B. oklahomensis* types as *B. pseudomallei*, and the *B. thailandensis*-like strain MSMB43 types as *B. mallei* (unpublished data).

Here we introduce a new more highly validated allelic discrimination assay, referred to as BurkDiff, to provide a higher level of specificity for accurate identification of *B. pseudomallei* and *B. mallei* and simultaneous differentiation when necessary. Alternatively, as these two species often occupy disparate niches under normal circumstances, BurkDiff can be used as a single-probe assay for definitive identification of *B. pseudomallei* or *B. mallei*. 

Abstract

A real-time PCR assay, BurkDiff, was designed to target a unique conserved region in the *B. pseudomallei* and *B. mallei* genomes containing a SNP that differentiates the two species. Sensitivity and specificity were assessed by screening BurkDiff across 469 isolates of *B. pseudomallei*, 49 isolates of *B. mallei*, and 390 isolates of clinically relevant non-target species. Concordance of results with traditional speciation methods and no cross-reactivity to non-target species show BurkDiff is a robust, highly validated assay for the detection and differentiation of *B. pseudomallei* and *B. mallei*.
### Table 1. Number and origin of *B. pseudomallei*, *B. mallei*, and genetic near-neighbor strains used in this study.

| Species       | Country      | Isolated from | No. of isolates | TaqMan result (SNP state) |
|---------------|--------------|---------------|-----------------|---------------------------|
| *B. mallei*   | China        | Human         | 2               | C                         |
|               | China        | Animal        | 4               | C                         |
|               | China        | Unknown       | 2               | C                         |
|               | France       | Unknown       | 1               | C                         |
|               | Hungary      | Animal        | 1               | C                         |
|               | Hungary      | Unknown       | 1               | C                         |
|               | India        | Animal        | 3               | C                         |
|               | India        | Unknown       | 1               | C                         |
|               | Pakistan     | Unknown       | 6               | C                         |
|               | Turkey       | Human         | 4               | C                         |
|               | Turkey       | Animal        | 1               | C                         |
|               | Turkey       | Unknown       | 10              | C                         |
|               | UK           | Unknown       | 1               | C                         |
|               | USA          | Human         | 4               | C                         |
|               | USA          | Animal        | 1               | C                         |
|               | USA          | Unknown       | 3               | C                         |
|               | Unknown      | Animal        | 2               | C                         |
|               | Unknown      | Unknown       | 2               | C                         |
| **Total**     |              |               | **8**           | **49**                    |

* B. pseudomallei

| Species       | Country      | Isolated from | No. of isolates | TaqMan result (SNP state) |
|---------------|--------------|---------------|-----------------|---------------------------|
|               | Australia    | Human         | 131             | A                         |
|               | Australia    | Animal        | 10              | A                         |
|               | Australia    | Environmental| 57              | A                         |
|               | Australia    | Unknown       | 6               | A                         |
|               | Bangladesh   | Human         | 2               | A                         |
|               | Cambodia     | Unknown       | 2               | A                         |
|               | China        | Unknown       | 3               | A                         |
|               | Ecuador      | Human         | 2               | A                         |
|               | Ecuador      | Animal        | 1               | A                         |
|               | Fiji         | Human         | 1               | A                         |
|               | India        | Unknown       | 1               | A                         |
|               | Indonesia    | Environmental| 1               | A                         |
|               | Kenya        | Human         | 1               | A                         |
|               | Kenya        | Environmental| 2               | A                         |
|               | Laos         | Unknown       | 2               | A                         |
|               | Madagascar   | Environmental| 2               | A                         |
|               | Malaysia     | Human         | 2               | A                         |
|               | Malaysia     | Environmental| 3               | A                         |
|               | Malaysia     | Unknown       | 15              | A                         |
|               | Mauritius    | Human         | 1               | A                         |
|               | Pakistan     | Human         | 2               | A                         |
|               | Papua New Guinea | Human   | 1               | A                         |
|               | Papua New Guinea | Unknown | 1               | A                         |
|               | Puerto Rico  | Human         | 2               | A                         |
|               | Singapore    | Human         | 2               | A                         |
|               | Singapore    | Environmental| 1               | A                         |
|               | Sweden       | Human         | 1               | A                         |
|               | Thailand     | Human         | 89              | A                         |
|               | Thailand     | Environmental| 105             | A                         |
|               | Unknown      | Human         | 1               | A                         |
|               | Unknown      | Environmental| 2               | A                         |
Methods

We used the methods described by Pearson et al. [21] to compare 25 B. pseudomallei and 10 B. mallei genomes to search for shared orthologous SNPs, then filtered them by mismatch value (the distance to the next SNP in bases). We further analyzed the resultant pool of SNPs and their flanking regions with a GenBank BLAST search, and finally chose one for assay development and validation.

Using Primer Express 3.0 software (Life Technologies, Foster City, CA), we designed a Taqman SNP dual-probe allelic discrimination assay in which one probe was designed to hybridize with the B. mallei allele (5‘-FAM-CTGAAACGCGGACGG-3’-MGB) and the other to the B. pseudomallei allele (5‘-VIC-CTGAAACGCGG-3’-MGB). Real-time PCR was carried out in 10 μL reactions containing 900 nM of each forward (5‘-CGAGCGCATCGTACTCGTA-3’) and reverse (5‘-CAAGT-CGGTTGAGTTGCCATTA-3’) primers, 200 nM of each probe, 1X Applied Biosystems Genotyping Mastermix, and 0.5 ng template. Thermal cycling and endpoint analysis was performed on an AB 7900HT sequence detection system (Life Technologies) using the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min.

To evaluate the utility of this SNP and its locus as a diagnostic marker for B. pseudomallei and B. mallei, we used the Taqman allelic discrimination assay to genotype a collection of human, animal, and environmental isolates of B. pseudomallei (n = 469) and B. mallei (n = 49) from a broad geographic range (Table 1). Additionally we assessed specificity by screening isolates of near-neighbor species (n = 469), and isolates of species of similar clinical presentation or restrictions imposed when animal glanders is diagnosed, but not scenarios in which the two species’ distinction is necessary. The trade would be used in clinical practice for diagnosis. However there are circumstances in which the two species for reporting purposes and forensic tracking as a bioterrorism agent both underscore the need to differentiate between the two species for reporting purposes and forensic tracking [22,23]. Rapid species identification may assist with appropriate initial patient treatment for human glanders. Currently physicians prescribe the lengthy drug regimen particular for melioidosis to inappropriately-treated B. pseudomallei-infected patients necessitate the rapid, specific identification of these species in the clinic [1,3]. The likelihood of infection with B. pseudomallei and B. mallei may not be equal given the circumstances; factors such as geographical distribution, prevalence, and risk factors for the diseases would be used in clinical practice for diagnosis. However there are scenarios in which the two species’ distinction is necessary. The trade restrictions imposed when animal glanders is diagnosed, but not animal melioidosis, and the potential use of B. pseudomallei or B. mallei as a bioterrorism agent both underscore the need to differentiate between the two species for reporting purposes and forensic tracking [22,23]. Rapid species identification may assist with appropriate initial patient treatment for human glanders. Currently physicians prescribe the lengthy drug regimen particular for melioidosis to human glanders patients [3], despite the differing in vitro antibiotic susceptibility profiles of the two causative agents [24].

Results

Genome comparisons revealed 1,235 SNPs with shared character states among all B. mallei genomes that differ from the character state shared by all B. pseudomallei genomes. Filtering the 1,235 SNPs using a mismatch value of 100 resulted in a pool of 74 SNPs. The GenBank BLAST search revealed the exclusivity of one of the regions to B. pseudomallei and B. mallei, so it was selected for assay development and validation.

Out of the isolates screened with BurkDiff, all 469 B. pseudomallei strains were shown to contain the allele with the SNP state A, and all 49 B. mallei strains were shown to contain that with the SNP state C (Table 1, Figure 1). No amplification of DNA from the 390 non-target species was detected, including the B. oklahomensis and the B. thailandensis-like strain MSMB43, both of which cross-reacted with a previously published allelic discrimination assay [20]. The limit of detection analysis showed consistent detection and allelic discrimination of B. pseudomallei and B. mallei at DNA template levels as low as 10^2 genome copies with sporadic amplification and genotyping at <10^2 genome copies (Figure 2).

Discussion

The universality of the clinical manifestations of human melioidosis and glanders precludes patient presentation as a definitive diagnostic for these diseases [3,22]. Diagnosis by traditional methods can be too time-consuming, or require special equipment [1]. The intrinsic resistance of B. pseudomallei and B. mallei to many widely-used antibiotics and the swift downward progression of untreated or inappropriately-treated B. pseudomallei- and B. mallei-infected patients necessitate the rapid, specific identification of these species in the clinic [1,3]. The likelihood of infection with B. pseudomallei and B. mallei may not be equal given the circumstances; factors such as geographical distribution, prevalence, and risk factors for the diseases would be used in clinical practice for diagnosis. However there are scenarios in which the two species’ distinction is necessary. The trade restrictions imposed when animal glanders is diagnosed, but not animal melioidosis, and the potential use of B. pseudomallei or B. mallei as a bioterrorism agent both underscore the need to differentiate between the two species for reporting purposes and forensic tracking [22,23]. Rapid species identification may assist with appropriate initial patient treatment for human glanders. Currently physicians prescribe the lengthy drug regimen particular for melioidosis to human glanders patients [3], despite the differing in vitro antibiotic susceptibility profiles of the two causative agents [24]. Of the rapid molecular methodologies with the capabilities of identifying and differentiating between B. pseudomallei and B. mallei, BurkDiff is unique in being single-step, single-reaction. In cases in which circumstances eliminate the possibility of one of the two species, BurkDiff can be used as a single-probe assay for specific identification.
The increasingly cosmopolitan nature of human activity inevitably exposes non-endemic area residents to *B. pseudomallei* and *B. mallei*, increasing the need for heightened awareness of these organisms outside their areas of endemicity, as has been demonstrated by numerous cases of imported melioidosis [25,26]. Most of these cases can be attributed to exposure during travel to tropical areas. However, global trade in commodities such as animals, plants and soils, and food items is also a possible transmission source resulting in disease in individuals with no travel history [27]. Diagnostic capabilities in non-endemic areas are becoming essential, not only for rapid, appropriate patient treatment, but for the safety of laboratory workers culturing the unknown organism for diagnosis.

### Table 2. Species and number of differential diagnostic and background flora strains screened across BurkDiff to validate the assay’s specificity.

| Species                              | No. of strains | Species                              | No. of strains |
|--------------------------------------|----------------|--------------------------------------|----------------|
| Abiotrophia/Granulicatella grp        | 1              | Neisseria gonorrhoeae                | 4              |
| Achromobacter xylosoxidans           | 1              | Neisseria meningitidis               | 3              |
| Acinetobacter baumannii              | 7              | Pasteurella multocida                | 1              |
| Bacillus anthracis                    | 1              |Providencia stuartii                 | 1              |
| Bacillus cereus                       | 1              | Pseudomonas aeruginosa               | 7              |
| Bacillus sp.                          | 2              | Rhotia mucilaginosa                  | 1              |
| Bacteroides fragilis                  | 1              | Salmonella enterica                  | 1              |
| Bacteroides uniformis                 | 1              | Shigella dysenteriae                 | 1              |
| Bordetella bronchiisepicia            | 1              | Staphylococcus arlettae              | 1              |
| Brucella abortus                      | 1              | Staphylococcus aureus                | 55             |
| Brucella suis                         | 1              | Staphylococcus capitis               | 1              |
| Candida albicans                      | 5              | Staphylococcus cohnii                | 1              |
| Candida glabrata                      | 2              | Staphylococcus epidermidis           | 8              |
| Candida parapsilosis                  | 3              | Staphylococcus equorum               | 1              |
| Candida tropicalis                    | 1              | Staphylococcus gallinarum            | 1              |
| Chryseobacterium indologenes         | 1              | Staphylococcus haemolyticus          | 3              |
| Coagulase negative Staphylococcus     | 16             | Staphylococcus hominis               | 1              |
| Coccioides immittis                   | 1              | Staphylococcus lugdunensis           | 1              |
| Coccioides posadasii                  | 2              | Staphylococcus saprophyticus         | 2              |
| Corynebacterium diptheriae            | 1              | Staphylococcus xenodochi             | 1              |
| Corynebacterium jeikeium              | 1              | Streptococcus agalactiae             | 9              |
| Coxiella burnetii                     | 2              | Streptococcus anginosus              | 2              |
| Enterobacter aerogenes                | 2              | Streptococcus equi                   | 1              |
| Enterobacter cloacae                  | 10             | Streptococcus gordonii               | 1              |
| Enterococcus faecalis                 | 9              | Streptococcus mitis                  | 2              |
| Enterococcus faecium                  | 6              | Streptococcus mutans                 | 1              |
| Escherichia coli                      | 11             | Streptococcus mutans                 | 1              |
| Francisella tularensis                | 2              | Streptococcus salivarius             | 2              |
| Haemophilus influenzae                | 4              | Streptococcus species                | 1              |
| Haemophilus parainfluenzae            | 2              | Streptococcus viridans grp           | 8              |
| Human gDNA                            | 2              | Vancomycin Resistant Enterococcus    | 4              |
| Klebsiella oxytoca                    | 1              | Yersinia pestis                      | 1              |
| Klebsiella pneumoniae                 | 8              | Yersinia pseudotuberculosis          | 1              |
| Lactococcus lactis                    | 1              | Total                                | 328            |
| Legionella pneumophila                | 1              |                                      |                |
| Listeria monocytogenes                | 1              |                                      |                |
| Micrococcus sp.                       | 1              |                                      |                |
| Moraxella catarrhalis                 | 7              |                                      |                |
| Mycobacterium avium                  | 1              |                                      |                |
| Mycoplasma pneumoniae                 | 1              |                                      |                |

Out of the 328 strains from approximately 80 species, none amplified. doi:10.1371/journal.pone.0015413.t002
Molecular assays, including BurkDiff, are rapid, sensitive, and specific, requiring only the appropriate thermal cycler and reagents common to many labs and obviating the need for direct culture of a dangerous pathogen. In fact, BurkDiff was successfully used among a panel of real-time assays targeting *B. pseudomallei* in the confirmation and characterization of a melioidosis case in an Arizona resident with no travel history [27].

Our use of whole genome sequence data allowed for targeted identification of phylogenetically informative markers (i.e., SNPs) to distinguish between *B. pseudomallei* and *B. mallei*, a preferred method to random identification of SNPs in conserved genes, as was done previously [20]. Additionally, *in silico* analyses of the markers allowed for the design of a highly specific assay. The illustrated specificity of BurkDiff to *B. pseudomallei* and *B. mallei* suggests that insertion of the genomic region that this assay targets occurred during or subsequent to the *B. pseudomallei*/*B. mallei* evolutionary split from its close genetic relative *B. thailandensis* [28]. Our data also suggest that the SNP targeted by BurkDiff is from a subsequent point mutation that occurred after the *B. mallei* lineage diverged from *B. pseudomallei* [28]. The number and diversity of the *B. pseudomallei* and *B. mallei* isolates successfully genotyped using BurkDiff suggest the genomic insertion is evolutionarily stable and

![Figure 1. BurkDiff allelic discrimination plot. Results from the assay across 45 *B. pseudomallei* and 23 *B. mallei* strains are shown, along with 2 no template controls (NTCs) and 26 near-neighbor and differential diagnostic species.](#)
A Real-Time PCR Assay for *Burkholderia* sp.
therefore a good target for identifying the species, while the point mutation could now be considered a canonical SNP (canSNP), a point mutation that marks a point of evolutionary divergence of two taxa and is inherently stable and thus definitive [29].

BurkDiff adds to the growing number of molecular based assays, especially real-time PCR, that have been designed to detect B. pseudomallei and/or B. mallei. Using several of these assays in combination for definitive identification could be important, as the Burkholderiaceae are highly recombining organisms [15,19,30], and as more and more strains are uncovered, the robustness and sensitivity of these assays will be challenged.

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Author Contributions
Conceived and designed the experiments: DME AT DMW BJC PSK. Performed the experiments: JRB JLG TP. Analyzed the data: JRB AT. Contributed reagents/materials/analysis tools: DME SJP AT DMW BJC PSK. Wrote the paper: JRB DME TP SJP AT.