Using real-time PCR to specifically detect *Burkholderia mallei*

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Burkholderia mallei is the causative agent of human and animal glanders and is a category B biothreat agent. Rapid diagnosis of *B. mallei* and immediate prophylactic treatment are essential for patient survival. The majority of current bacteriological and immunological techniques for identifying *B. mallei* from clinical samples are time-consuming, and cross-reactivity with closely related organisms (i.e. *Burkholderia pseudomallei*) is a problem. In this investigation, two *B. mallei*-specific real-time PCR assays targeting the *B. mallei* *bimA_{ma}* gene (*Burkholderia* intracellular motility A; BMAA0749), which encodes a protein involved in actin polymerization, were developed. The PCR primer and probe sets were tested for specificity against a collection of *B. mallei* and *B. pseudomallei* isolates obtained from numerous clinical and environmental (*B. pseudomallei* only) sources. The assays were also tested for cross-reactivity using template DNA from 14 closely related *Burkholderia* species. The relative limit of detection for the assays was found to be 1 pg or 424 genome equivalents. The authors also analysed the applicability of assays to detect *B. mallei* within infected BALB/c mouse tissues. Beginning 1 h post aerosol exposure, *B. mallei* was successfully identified within the lungs, and starting at 24 h post exposure, in the spleen and liver. Surprisingly, *B. mallei* was not detected in the blood of acutely infected animals. This investigation provides two real-time PCR assays for the rapid and specific identification of *B. mallei*.

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INTRODUCTION

Burkholderia mallei, the aetiologic agent of glanders, is a Gram-negative, non-motile bacillus that is a highly adapted obligate parasite of equines and is unable to persist outside of its host (DeShazer & Waag, 2004). Until the early twentieth century and the development of motorized transportation, glanders was common throughout the world (DeShazer & Waag, 2004). With the development of the mallein test (a serological assay for identifying *B. mallei*-infected horses), there have not been any naturally occurring cases of human glanders reported in the USA since the 1930s. However, sporadic cases of glanders still occur in Asia, Africa, the Middle East, and South America. Human glanders is now uncommon, and individuals such as veterinarians, slaughterhouse workers and laboratory scientists, whose occupation exposes them to infection, are most susceptible to acquiring glanders. In horses, clinical symptoms of glanders include nasal discharge, possible ulceration of the nasal septum, enlarged lymph nodes, and characteristic ulcers, pustules and nodules in the lungs and on the skin (DeShazer & Waag, 2004).

Abbreviations: C_T value, crossing threshold value; EPF, end-point fluorescence; MGB probe, minor groove binding probe; p.e., post exposure.

Acute human glanders is normally fatal without immediate antibiotic treatment. Infected individuals may present clinically with swollen lymph nodes, ulcerating nodules in the alimentary and respiratory tracts, and weight loss, as well as numerous subcutaneous abscesses (DeShazer & Waag, 2004; Srinivasan *et al.*, 2001). The route of exposure for human glanders is normally via contact with infectious material (i.e. nasal discharge from infected horses) through skin abrasions or cuts, which usually results in a chronic form of disease. In contrast, and likely the route of exposure in the deliberate release of *B. mallei*, contraction from aerosols causes acute glanders, and if not treated immediately, death occurs in a matter of days (Howe & Miller, 1947; Hunter, 1936).

With the potential of *B. mallei* weaponization, rapid methods for the identification of this infectious *Burkholderia* species are essential to ensure that adequate prophylactic therapies can be initiated following exposure. The conventional laboratory methods for detecting *B. mallei* rely on biochemical testing, colony morphology, and motility assays, all of which can take up to 7 days to obtain preliminary results. In addition, it has recently been demonstrated that the API 20NE and RAPID NF commercial kits are 0–60 % successful in positively identifying *B. mallei* (Glass & Popovic, 2005).

With the physiological and genetic relatedness between *B. mallei*, *Burkholderia pseudomallei* and *Burkholderia thailandensis* (Brett *et al.*, 1997; Godoy *et al.*, 2003; Holden *et al.*, 2004; Nierman *et al.*, 2004), PCR-based and biochemical techniques for identifying and differentiating these *Burkholderia* species are difficult. For *B. pseudomallei*, the 16S and 23S rRNA, 16S–23S intergenic region, *fliC*, heat-shock protein 70 (Hsp70), and a type three secretion (TTS) system have been targeted for the development of molecular-based assays (Antonov *et al.*, 2005; Bauernfeind *et al.*, 1998; 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.*, 2004, 2005; Tyler *et al.*, 1995; U'Ren *et al.*, 2005). One study required the use of two separate assays and a negative PCR result to discriminate *B. mallei* from *B. pseudomallei*. Several other investigations could differentiate *B. pseudomallei* from *B. mallei*, but these reports were based on a single base pair difference between the two species. The lack of a target region with substantial nucleotide variation between *B. pseudomallei* and *B. mallei* further demonstrates the genetic similarity between these *Burkholderia* species.

In this investigation, we have designed a real-time PCR assay for the definitive identification of *B. mallei* and separation from *B. pseudomallei*. The assay was tested for specificity against a panel of diverse bacterial species, and for the ability to detect *B. mallei* within the lungs, liver and spleen of aerosol-challenged BALB/c mice.

METHODS

Bacterial culture conditions. All *B. mallei* isolates were cultured using Luria–Bertani (LB) broth containing 4% (v/v) glycerol (Sigma) (LBG) at 37 °C with aeration (250 r.p.m.). The remaining *Burkholderia* isolates were cultured as described above using LB broth. A representative panel of eight *B. mallei*, eight *B. pseudomallei* and eight *B. thailandensis* strains, obtained from various geographical, clinical and environmental locations throughout the world, was investigated (Table 3). Additional closely related *Burkholderia* species tested included *Burkholderia cepacia*, *Burkholderia stabilis*, *Burkholderia multivorans* and *Burkholderia vietnamiensis*. Other bacterial isolates tested for cross-reactivity are described below.

***B. mallei* animal challenges and tissue collection.** Female BALB/c mice, 6–8 weeks old, were obtained from Charles River Laboratories (National Cancer Institute, Frederick, MD) and challenged by the aerosol route with approximately 10 LD₅₀ of *B. mallei* ATCC 23344. Briefly, whole-body exposures were performed on 20 mice by nebulizing a 10 ml overnight culture of *B. mallei* ATCC 23344 which delivers an inhaled dose of approximately 1000 c.f.u., or 10 LD₅₀. At 1, 24 and 48 h following exposure, three mice for each time-point were euthanized (CO₂ chamber) and lungs (1, 24 and 48 h), spleens and livers (24 and 48 h) were harvested. Individual organs were placed in scintillation tubes containing 1 ml sterile PBS and organs were disrupted using a Tissue-Tearor (BioSpec Products) by administering 20 s pulses (3×) on setting thirty or until the tissue was appropriately homogenized. Organ bacterial loads were determined by serially diluting each organ extract (100 µl tissue into 900 µl PBS), followed by cultivation on LBG plates at 37 °C for 48 h. For blood collection, three animals (same mice used for organ extractions) at each time-point were exsanguinated by intracardial puncture. All research was conducted

in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Bacterial and tissue DNA extraction. *Burkholderia* species were cultured as described above, and genomic DNA from cultures as well as collected tissues was prepared using the Qiagen QIAamp DNA Mini kit with 180 µl of an overnight culture or homogenized tissue, with the following modifications to the manufacturer's protocol. The sample (180 µl) was mixed with 20 µl proteinase K (17.8 mg ml⁻¹) and 200 µl buffer AL, and incubated at 55 °C for 1 h. Ethanol (96–100%, 210 µl) was added and mixed, and the sample was loaded onto the column and the column washed using buffers AW1 and AW2. Columns were centrifuged at 16 000 g for 3 min to dry residual ethanol from the column. Buffer AE (100 µl), preheated to 70 °C, was added to each column, incubated at 70 °C for 5 min, and DNA was eluted.

The DNA used in this study for cross-reactivity testing was from reference material obtained from recognized culture collections, commercial vendors, clinics, or unique entries from previous United States Army Medical Research Institute of Infectious Diseases (USAMRIID) collections. Pure cultures of each agent were grown on appropriate media at optimal temperatures and harvested for DNA isolation. The DNA was extracted using either Bactozol Kits (Molecular Research Center) according to the manufacturer's recommendations or Qiagen kits as previously described (Coyne *et al.*, 2004). Extracted DNA was quantified and checked for purity with a spectrophotometer by measuring absorbance at 260, 280 and 320 nm. The ability of the DNA to be amplified was established with a universal primer set to the 16S rRNA gene (Bricker & Halling, 1994). The presence of high-molecular-mass DNA was confirmed by running 400 ng ml⁻¹ of each genomic DNA on 0.7% agarose gels. Identity was confirmed for each DNA by sequence analysis of the entire 16S rRNA gene and comparison with published data.

Assay design and optimization. The real-time PCR assay primer and probe sequences are listed in Table 1. The *B. mallei* *bimA*_{ma} (*Burkholderia* intracellular motility A) homologue encoded by gene locus BMAA0749 was used for assay development. It has recently been demonstrated that the N-terminal nucleotide sequence of *bimA*_{ma} contains a unique *B. mallei* region not present in the *B. pseudomallei* and *B. thailandensis* *bimA* genes (Stevens *et al.*, 2005a). The specific primer and TaqMan minor groove binding (MGB) probe sequences were designed using Visual OMP version 4.1.0.0 for Windows (DNA Software). All primer and probe sequences were analysed using BLASTN to ensure specificity for their respective targets. All primers were purchased from Invitrogen. The probes were synthesized by Applied Biosystems, and contained 6-carboxyfluorescein (FAM) at the 5' end and a non-fluorescent quencher with the MGB protein at the 3' end. Primer candidates were used to amplify 10 pg target template in the presence of SYBR green dye. Melt curves and agarose gels were analysed to eliminate inefficient and/or dimer-producing primer pairs. MgCl₂ and primer concentrations were optimized sequentially as follows: MgCl₂ was optimized in 1 mM increments from 3 to 7 mM, and primers were optimized symmetrically in 0.1 µM increments from 0.1 to 1.0 µM. The combinations that exhibited the earliest crossing threshold (C_T) value and generated the highest end-point fluorescence (EPF) value were chosen as the optimal conditions for each assay (Table 1).

The optimized assays were carried out in 20 µl volumes. Each assay contained 1× PCR buffer (50 mM Tris, pH 8.3, 250 µg BSA ml⁻¹) (Idaho Technology, Inc.), 0.2 mM dNTP mix (Idaho Technology,

Table 1. Primer and probe sequences for *B. mallei*-specific real-time PCR assay

Amplicon sizes, sequences and optimized conditions are shown.

Assay	Target	Amplicon size (bp)	Primer/probe	Sequence	Final concn	MgCl ₂ concn
F49R144	<i>bimA</i> _{ma}	96	BMSEC3-F49	CAGTTGATTCTCCCACC	0.5 mM	5 mM
			BMSEC3-R144	TGTCTTGTGAGCATGAGA	0.5 mM	
			MGB probe BMSEC3-P104R	CATACGGATGTATAGAACCAAT	0.15 mM	
F52R146	<i>bimA</i> _{ma}	95	BMSEC3-F52	TTGATTCTCCCACCCGA	0.5 mM	5 mM
			BMSEC3-R146	GGTGTCTTGTGAGCAT	0.5 mM	
			MGB probe BMSEC3-P104R	CATACGGATGTATAGAACCAAT	0.15 mM	

Inc.) and 1.0 U Platinum *Taq* DNA polymerase (Invitrogen). Optimal concentrations of primers, probe and MgCl₂ were added, and the master mix was distributed to reaction tubes. Five microlitres of control/template/sample DNA was added just before analysis on the instrument. Thermal cycling conditions were standardized for both assays, and consisted of 1 cycle at 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 20 s. Fluorescence readings were taken at the end of each 60 °C step. For the LightCycler 2.0 instrument (Roche), each reaction capillary tube was read in channel 1 (F1) with data analysed by the LightCycler Data Analysis (LCDA) software version 4.0.

Assay linearity and estimated detection limit. The linearity of the assay was assessed by standard curve analysis in order to determine the amplification efficacy, efficiency, and quantitative utility. Additionally, serial dilutions were used to estimate the limit of detection. Serial dilutions in Tris/EDTA buffer (Eppendorf) were prepared at the following per-reaction concentrations: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg. Triplicate assays were performed at each dilution, the standard curve was generated by the LightCycler 4.0 software, and slopes were used to calculate amplification efficiency and standard error. An estimate of the detection limit was determined by the lowest concentration of template per reaction that produced three positive results.

Inclusivity and exclusivity experiments. A general panel of 60 organisms was analysed using the optimized conditions to establish inclusivity and exclusivity (Table 2). In addition, a panel of 30 genetic near neighbours was also analysed (Table 3). Panels were constructed to include: other threat organisms; nearest genetic neighbours to threat organisms; organisms sharing a clinical niche with *B. mallei*, especially respiratory pathogens, opportunists, and normal respiratory flora; organisms observed repeatedly in clinical and environmental samples. In all cases, 100 pg genomic DNA per reaction was used to determine if the assays cross-reacted with nucleic acid from other organisms. Inclusivity and exclusivity testing was performed on the LightCycler 2.0 instrument and utilized the automated calling capability of LightCycler software version 4.0.

RESULTS

Identification of a *B. mallei* unique genomic DNA sequence

Stevens *et al.* (2005b) have recently identified a putative protein (BimA) synthesized by *B. pseudomallei* that is involved in host-cell actin polymerization. *In silico* analysis of the *B. mallei* and *B. thailandensis* genomes indicates that these *Burkholderia* species also encode a BimA orthologue.

Interestingly, considering that the *B. mallei* and *B. thailandensis* (a closely related avirulent *Burkholderia* species) BimA proteins are capable of complementing a *B. pseudomallei* *bimA* mutant, significant nucleotide sequence deviations have been identified within the N-terminal coding regions of this allele (Stevens *et al.*, 2005a). Using this unique DNA sequence within the 5' region of the *B. mallei* *bimA*_{ma}, two real-time PCR primer and probe sets (Table 1) were designed and tested for the capability to detect *B. mallei* within infected lungs, spleens and livers of challenged BALB/c mice. Cross-reactivity to eight *B. pseudomallei* isolates and eight *B. thailandensis* strains obtained from various geographical, clinical and environmental sources over a 70 year period was tested.

Assay optimization

Primers were designed from a portion of the 5' region of the *B. mallei* *bimA*_{ma} sequence and initially tested by rapid PCR amplification in the presence of SYBR Green. All primer combinations resulting in PCR products smaller than 160 bp were tested for amplification efficiency. Initial testing was performed with five forward and five reverse primers in a matrix that produced 13 combinations. All combinations amplified *B. mallei* DNA from strain ATCC 23344. Two combinations, F49 with R144 and F52 with R146, generated the largest amount of amplified DNA. In addition, no template controls with these two primer sets produced minimal to no primer dimers. After primers were tested and selected using standard PCR, five compatible probes were tested for performance with both of these primer sets. For both primer sets, probe P104R consistently produced the earliest C_T and the highest EPF values. Assays were further optimized for primer and MgCl₂ concentrations to produce maximum sensitivity, earliest C_T and highest EPF. The final primer and probe pairs and optimized assay conditions are listed in Table 1.

Sensitivity/efficiency

Standard curve analysis was utilized to assess the amplification efficiency and approximate detection limit of the assays. Standard curves were designed to contain triplicate samples at a minimum of six detectable concentrations of DNA. The

Table 2. DNAs that encompass the general cross-reactivity panel analysed in this investigation

All assays were performed with 100 pg template DNA per reaction from each organism to ensure specificity. NA, Not applicable.

Organism	ATCC no.	Product no.*
<i>Acinetobacter baumannii</i>	19606	ACIN001-DP15
<i>Actinomyces naeslundii</i>	12104D	ACTM001D-ATCC
<i>Actinobacillus pleuropneumoniae</i>	27088D	ACTN001D-ATCC
<i>Alcaligenes xylosoxydans</i>	27061	ALCA001-BZ009
<i>Bacillus anthracis</i> Ames	NA	BACI008-BZ021
<i>Bacillus stearothermophilus</i>	7953	BACI032-BZ015
<i>Bacillus thuringiensis</i>	35646	BACI036-DP12
<i>Bacillus subtilis</i> var. <i>niger</i>	NA	BACI051-BZ005
<i>Bacillus cereus</i>	19637	BACI172-BATT03
<i>Bacteroides fragilis</i>	25285D	BACT002D-ATCC
<i>Bartonella henselae</i>	49882D	BART001D-ATCC
<i>Bifidobacterium infantis</i>	15697D	BIFI003D-ATCC
<i>Bordetella bronchiseptica</i>	10580	BORD001-BZ019
<i>Bordetella pertussis</i>	9797D	BORD003D-ATCC
<i>Borrelia burgdorferi</i>	35210D	BORR001D-ATCC
<i>Brucella melitensis</i>	NA	BRUC013-AFIP01
<i>Budvicia aquatica</i>	35567	BUDV001-BZ017
<i>Burkholderia pseudomallei</i>	NA	BURK003-BZ044
<i>Campylobacter jejuni</i>	33560D	CAMP002D-ATCC
<i>Candida albicans</i>	10231D	CAND001D-ATCC
<i>Chryseobacterium meningosepticum</i>	33958D	CHRY001D-ATCC
<i>Clostridium botulinum</i> type A	19397	CLOS001-QM10
<i>Clostridium difficile</i>	9689D	CLOS038D-ATCC
<i>Clostridium perfringens</i>	13124	CLOS004-BZ026
<i>Comamonas acidovorans</i>	15668	COMA003-DP12
<i>Corynebacterium diphtheriae</i>	700971D	CORY005D-ATCC
<i>Coxiella burnetii</i>	NA	COXI001-QM017
<i>Deinococcus radiodurans</i>	13939D	DEIN001D-ATCC
<i>Enterobacter aerogenes</i>	NA	ENTB001-BATT03
<i>Enterobacter agglomerans</i>	29904	ENTB002-BATT03
<i>Enterococcus faecalis</i>	700802D	ENTC006D-ATCC
<i>Escherichia coli</i>	25922	ESCH001-BZ001
<i>Francisella tularensis</i>	NA	FRAN016-BZ43
<i>Haemophilus influenzae</i>	10211	HAEM001-DP11
Human DNA	NA	HUMA001-NOVA00
<i>Klebsiella pneumoniae</i>	13883	KLEB001-DP12
<i>Legionella pneumophila</i>	33152D	LEGN001D-ATCC
<i>Listeria monocytogenes</i>	15313	LIST001-BZ035
<i>Moraxella catarrhalis</i>	25240	MORA001-BZ013
<i>Mycoplasma pneumoniae</i>	15531D	MYCO001D-ATCC
<i>Neisseria meningitidis</i>	53415D	NEIS007D-ATCC
<i>Pasteurella multocida</i>	43137	PAST001-BATT03
<i>Porphyromonas gingivalis</i>	33277D	PORP002D-ATCC
<i>Proteus mirabilis</i>	7002	PROT001-DP15
<i>Pseudomonas aeruginosa</i>	17933D	PSEU004D-AFIP
<i>Rhizobium radiobacter</i>	33970D	RHIZ001D-ATCC
<i>Salmonella choleraesuis</i> subsp. <i>choleraesuis</i> serotype <i>Paratyphi</i>	9150D	SALM004D-ATCC
<i>Serratia marcescens</i>	13880	SERR003-BATT03
<i>Shigella flexneri</i>	12022	SHIG001-DP10
<i>Staphylococcus aureus</i>	29247	STAP014-BATT02

Table 2. cont.

Organism	ATCC no.	Product no.*
<i>Staphylococcus epidermidis</i>	12228D	STAP039D-ATCC
<i>Stenotrophomonas maltophilia</i>	13637	STEN001-BZ032
<i>Streptococcus pyogenes</i>	19615	STRE010-DP11
<i>Streptococcus pneumoniae</i>	33400	STRE017-DP10
<i>Ureaplasma urealyticum</i>	700970D	UREA001D-ATCC
Vaccinia virus	NA	VACC001-CDC
<i>Vibrio cholerae</i>	51394D	VIBR002D-ATCC
<i>Yersinia pseudotuberculosis</i>	6904	YERS008-DP13
<i>Yersinia enterocolitica</i>	NA	YERS014-DP14
<i>Yersinia pestis</i> CO92	NA	YERS023-BZ023

*Product number corresponds to unique identifiers for United States Army Medical Research Institute of Infectious Diseases (USAMRIID) tracking purposes.

assays had amplification efficiencies of 1.97 (F49/R144) and 1.92 (F52/R146) with errors of 0.018 and 0.057, respectively. An amplification efficiency of 2.00 is considered ideal, and corresponds to a doubling of copy number for every PCR cycle. Both assays detected all three replicates at 1 pg DNA template per reaction, which corresponds to 424 genome copies, based on a genome size of 2.3 Mbp (this corresponds to *B. mallei* ATCC 23344 chromosome 2).

Inclusivity/exclusivity

Using standard PCR (Epicentre FailSafe kit with buffer J, Epicentre Technologies), with primers targeting the unique 5' DNA sequence of the *B. mallei* *bimA*_{ma} (AT5, 5'-TTTCGATCGATTCTGCTATC-3', and AT4, 5'-GCGT-TAAACGCCGTACTTTC-3'), we have previously tested a collection of 29 *B. mallei* and 34 *B. pseudomallei* isolates to determine if this unique *B. mallei* nucleotide sequence is conserved (Ulrich *et al.*, 2006). Surprisingly, given the extensive genetic similarity between *B. mallei* and *B. pseudomallei* (Godoy *et al.*, 2003), only *B. mallei* genomic DNA produced a detectable amplicon with PCR primers (listed above) specifically designed for *bimA*_{ma}. Likewise, both real-time PCR assays (Table 1) were analysed for their ability to specifically detect strains of *B. mallei* in the absence of non-specific detection of both genetic near neighbours as well as a broader collection of micro-organisms. The near-neighbour analysis included a panel of 30 DNAs, including eight strains of *B. thailandensis*, eight strains of *B. mallei*, eight strains of *B. pseudomallei* and six strains of other *Burkholderia* species, including *B. cepacia*, *B. multivorans*, *B. stabilis* and *B. vietnamiensis*. As observed with primer pairs AT4 and AT5 (standard PCR primers targeting *bimA*_{ma}), both assays detected only *B. mallei*, and consistent C_T values were observed across all strains and assays tested (Table 3). Both assays were negative for other genetic near neighbours, as well as for a panel of 60 micro-organisms from a general microbial DNA panel (Tables 2 and 3).

In vivo analysis

To determine if our real-time PCR assays were capable of detecting *B. mallei* within infected tissues and blood, we challenged (10 LD₅₀ or 1000 c.f.u.) 20 female BALB/c mice via the aerosol route and followed organ colonization for 48 h post exposure (p.e.). At 1 h p.e., our real-time PCR primer and probe sets definitively identified *B. mallei* within the lungs of mice, indicated by mean C_T values of 30.06 for primer pair 49F/144R and 30.15 for set 52F/146R (Table 4). At 24 h p.e., dissemination of *B. mallei* to the liver was detected in two out of three animals with primer pairs 49F/144R (mean C_T of 34.56) and 52F/146R (mean C_T of 35.19) (Table 4). Within the spleen, primers 52F/146R detected *B. mallei* in two out of three animals (mean cycle value of 35.57), whereas primers 49F/144R only detected *B. mallei* in one out of the three animals (C_T of 33.42) at 24 h p.e. (Table 4). At 48 h post challenge, all organ extracts (lungs, livers and spleens) tested positive for *B. mallei* (Table 4). The mean C_T values for the triplicate lungs were 23.50 for primer pair 49F/144R and 23.59 for primer pair 52F/146R. For extracted livers, the mean C_T values were 28.50 and 28.82 for 49F/144R and 52F/146R, respectively, and for spleen tissue, 31.56 with pair 49F/144R and 31.60 with set 52F/146R. It should be noted that BALB/c mice challenged by the aerosol route with *B. mallei* develop acute glanders and normally succumb to infection 48–72 h post challenge; therefore, additional time-points were not analysed. All control organ extracts, from non-infected animals housed within the same biosafety level 3 containment laboratory, were negative for the presence of *B. mallei* by analysis with the real-time PCR primers and probes described in this study (Table 4). Surprisingly, and likely a result of the low bacterial burden in the blood, our real-time PCR assays were not capable of detecting *B. mallei* in the blood of infected mice (Table 4). The presence of PCR inhibitors in all sample eluates was tested by using an inhibition assay previously described (Hartman *et al.*, 2005). The extracted DNA material from whole blood required a

Table 3. Near-neighbour analysis for the two *B. mallei*-specific real-time PCR assays

C_T values are provided for those strains that produced positive results for each assay. UN, Unknown information; Neg, negative assay result.

Organism and strain	Source	Location	Year	C _T value for assay:	
				Probe 104R-MGB, primers 49F/144R	Probe 104R-MGB, primers 52F/146R
<i>B. mallei</i>					
ATCC 23344	Human	China	1942	30·30	29·90
Turkey 1	UN	Turkey	UN	31·20	31·16
Turkey 8	UN	Turkey	UN	30·81	30·89
Turkey 10	UN	Turkey	UN	30·36	30·65
NCTC 10229	UN	Hungary	1961	30·37	30·85
2000031065	UN	Turkey	UN	30·50	30·87
2002721273	UN	USA	1956	30·89	31·03
2002721776	UN	USA	1956	30·36	30·03
<i>B. pseudomallei</i>					
K96243	Human	Thailand	1996	Neg	Neg
238	Blood	Australia	UN	Neg	Neg
316c	Blood	Thailand	1987	Neg	Neg
439a	Ulcer	Australia	UN	Neg	Neg
487	Ulcer	Australia	UN	Neg	Neg
E25	Soil	Thailand	UN	Neg	Neg
STW 176	Water	Thailand	UN	Neg	Neg
USAMRU 32	UN	Malaysia	1964	Neg	Neg
<i>B. thailandensis</i>					
E254	UN	UN	UN	Neg	Neg
E256	UN	Thailand	1993	Neg	Neg
E257	Soil	Thailand	UN	Neg	Neg
E260	Soil	Thailand	1993	Neg	Neg
E264	Environment	Thailand	1993	Neg	Neg
E266	Soil	Thailand	1993	Neg	Neg
E267	Soil	Thailand	1993	Neg	Neg
E290	Soil	Thailand	1993	Neg	Neg
<i>Burkholderia</i> species					
<i>Burkholderia</i> species 275	UN	UN	UN	Neg	Neg
<i>B. cepacia</i> ATCC 25416	Plant	UN	UN	Neg	Neg
<i>B. cepacia</i> K56	Clinical	Canada	1998	Neg	Neg
<i>B. multivorans</i>	UN	UN	UN	Neg	Neg
<i>B. stabilis</i>	UN	UN	UN	Neg	Neg
<i>B. vietnamiensis</i>	UN	UN	UN	Neg	Neg

dilution of 1:8 to overcome the effects of PCR inhibitors present in the sample (Table 4).

DISCUSSION

Rapid identification of *B. mallei* in the mammalian host is essential for the management of disease progression and prevention of acute glanders. Standard laboratory methods for the diagnosis of *B. mallei* from clinical samples focus on biochemical and phenotypical testing that can take in excess of 7 days (DeShazer & Waag, 2004), which is inadequate for potential acute cases of glanders (patients normally die 2 days p.e.). With the potential weaponization and

deliberate release of *B. mallei* into the public domain, rapid molecular methods for the identification of this highly infectious pathogen are crucial.

As previously mentioned, the current laboratory methods for identifying *B. mallei* in clinical samples rely on biochemical and phenotypical testing. However, it has been shown that commercially available biochemical testing kits (API 20NE and RapID NF) cross-react with other non-virulent bacterial species, leading to false-positive reactions (Glass & Popovic, 2005; Inglis *et al.*, 1998). An ELISA has been developed using irradiation-killed *B. mallei* whole cells; however, cross-reactivity with irradiation-killed *B. pseudomallei* was observed, suggesting that this

Table 4. Analysis of *B. mallei*-specific real-time PCR assays on infected mouse tissues

Neg, negative assay result.

Tissue	C _T value for assay:	
	Probe 104R-MGB, primers 49F/144R	Probe 104R-MGB, primers 52F/146R
Control lung	Neg	Neg
Control liver	Neg	Neg
Control spleen	Neg	Neg
Control blood	Neg	Neg
Lung		
1 h 1*	30·55	30·72
1 h 2*	29·94	30·04
1 h 3*	29·69	29·68
24 h 1	27·54	27·27
24 h 2	27·01	27·09
24 h 3	26·61	27·13
48 h 1	23·88	24·11
48 h 2	23·61	23·57
48 h 3	23·00	23·09
Liver		
24 h 1	34·80	35·33
24 h 2	Neg	Neg
24 h 3	34·31	35·04
48 h 1	28·16	28·67
48 h 2	28·99	29·42
48 h 3	28·35	28·36
Spleen		
24 h 1	Neg	35·61
24 h 2	33·42	Neg
24 h 3	Neg	35·53
48 h 1	32·32	32·88
48 h 2	30·95	30·96
48 h 3	31·40	30·96
Blood		
24 h 1 1:8	Neg	Neg
24 h 2 1:8	Neg	Neg
24 h 3 1:8	Neg	Neg
48 h 1 1:8	Neg	Neg
48 h 2 1:8	Neg	Neg
48 h 3 1:8	Neg	Neg

*1, Animal one; 2, animal two; 3, animal three.

immunological assay is not *B. mallei* specific (unpublished results).

The development of PCR-based methodologies for the identification and differentiation of *B. mallei* from *B. pseudomallei* and *B. thailandensis* has been troublesome due to the level of genetic similarity between these *Burkholderia* species (Brett *et al.*, 1998; Godoy *et al.*, 2003; Holden *et al.*, 2004; Nierman *et al.*, 2004). Various chromosomal markers have been used to detect *B. pseudomallei*, and include the

16S and 23S rRNA, 16S–23S intergenic region, the filament-forming flagellin *fliC* gene, heat-shock protein Hsp70, and a putative type three secretion operon (Antonov *et al.*, 2005; Bauernfeind *et al.*, 1998; Gee *et al.*, 2003; Hagen *et al.*, 2002; Lee *et al.*, 2005; Sprague *et al.*, 2002; Thibault *et al.*, 2004; Tomaso *et al.*, 2004, 2005; Tyler *et al.*, 1995; U'Ren *et al.*, 2005). Several of the assays that have been described are targeted toward single-nucleotide differences in the 23S rDNA region, the 16S region, and a putative antibiotic resistance gene. However, targeting a single base pair could be problematic for the identification of *B. mallei*, as it has been shown that the *B. mallei* genome is unstable upon passage. *B. mallei* ATCC 23344 has been passaged in culture, in a mouse and in a horse, and two isolates have been obtained from the accidental infection of a scientist (Srinivasan *et al.*, 2001). The genomes of these isolates were sequenced and compared to the published *B. mallei* sequence. Single-nucleotide polymorphisms were noted after passage, mainly located within coding regions of the genome. The two human isolates obtained from the accidental infection each possessed a distinct set of sequence variations as well as two sequence variations in common (unpublished data). These data suggest that the *B. mallei* population in a host is not identical and that accumulated genome-sequence variation may occur.

The *B. mallei*-specific real-time PCR assay described in this study was designed using a unique 5' nucleotide region encoded within *bimA*_{ma} (BMAA0749) (Stevens *et al.*, 2005a). In *B. pseudomallei*, BimA has been shown to be involved in host-cell actin polymerization (Stevens *et al.*, 2005b). Surprisingly, the unique DNA sequence encoding the N-terminal region of BimA_{ma}, the portion that is exposed at the bacterial cell surface, is conserved among numerous *B. mallei* isolates (29 strains obtained from various clinical sources over a 70 year period; Ulrich *et al.*, 2006), suggesting that this sequence divergence occurred early in the evolution of *B. mallei* from *B. pseudomallei*. These findings also suggest that this chromosomally encoded gene is genetically stable and will provide an ideal target for the rapid identification of *B. mallei*. Despite these deviations in nucleotide sequence, heterologous expression of the *B. mallei* *bimA*_{ma} in a *B. pseudomallei* *bimA*_{ps} mutant fully complements the defective actin motility phenotype observed in this strain, suggesting that *bimA*_{ma} encodes a functional protein (Stevens *et al.*, 2005a).

The specificity of our *B. mallei* real-time PCR assays was tested against an extensive cross-reactivity panel comprising bacterial (Gram-positive and -negative species), viral, fungal and human DNAs (Table 2). In addition to the cross-reactivity panel, a set of 30 closely related *Burkholderia* species was tested, including *B. mallei*, *B. pseudomallei*, *B. thailandensis*, *B. stabilis*, *B. multivorans*, *B. cepacia* and *B. vietnamiensis* (Table 3). With our real-time PCR primer and probe sets, *B. mallei* was definitively identified and differentiated from *B. pseudomallei* and other closely related species (Table 3).

To further test the applicability of our *B. mallei*-specific real-time PCR assay, serial animal sacrifices were performed on BALB/c mice infected by the aerosol route with *B. mallei* ATCC 23344. As anticipated, *B. mallei* was positively detected in organ extracts processed at 1 h (lungs), 24 h (lungs, spleen and liver) and 48 h p.e. (lungs, spleen and liver) (Table 4). The data produced in this study indicate the proliferation of bacterial cells in the organs of infected mice based on the decreasing C_T values for each of the time-points tested. These findings suggest that the real-time PCR primer and probe sets described in this work will provide a rapid method for the identification of *B. mallei* in clinical samples. Unlike previously described techniques for detecting *B. mallei* in human clinical samples (i.e. culturing abscess biopsies, GLC of cellular fatty acids, and 16S rRNA gene-sequence analysis), real-time PCR provides an alternative method for identifying *B. mallei* and eliminates the need for bacterial pre-enrichment (i.e. total DNA can be isolated from organ abscesses and real-time PCR performed). Unfortunately, the sensitivity (see below) of our *B. mallei*-specific real-time PCR assays was not sufficient to detect *B. mallei* in the blood of infected animals. These findings are likely the result of either the low bacterial load found within the blood of acutely infected BALB/c mice or the presence of PCR inhibitors (Fritz *et al.*, 2000). Even when incorporating spiked BALB/c control blood with *B. mallei* at concentrations of 1000, 500 and 100 c.f.u. ml⁻¹, we failed to identify this pathogen using the primer and probe sets described in this work (data not shown). However, we were able to detect *B. mallei* in spiked human blood (human use protocol FY04-14) at a concentration of 500 c.f.u. ml⁻¹ with primer set 49F/144R (three out of three replicates) (data not shown). These findings suggest that, despite using a commonly employed commercial DNA extraction kit (Qiagen QIAamp DNA Mini kit), mouse blood contains PCR inhibitors not present in human blood. Further studies to address these extraction limitations will be needed. Similar reductions in the ability to detect *B. mallei* and *B. pseudomallei* in spiked blood have been reported by Tomaso *et al.* (2005), who targeted the *B. pseudomallei* and *B. mallei* 16S rDNA, *fliC* and *rpsU* genes.

For serological detection of *B. mallei*, the Russian scientists Gelman and Kalning developed the mallein assay in the late 1800s (DeShazer & Waag, 2004). This immunological test utilized antigens prepared from 4–8 month culture filtrates of *B. mallei* and was utilized by the USA and Canada to identify horses infected with *B. mallei* (Steele, 1979). Despite the rapid (48 h) identification of *B. mallei* using the mallein test in horses, serological conversion does not occur in humans for 3–4 weeks p.e., which suggests that this assay is ineffective for detecting human glanders. It should be noted that this serological test also cross-reacts with *B. pseudomallei* (DeShazer & Waag, 2004). To date, the only *B. mallei*-specific detection assay reported in the literature incorporates the bacterial phage phiE125, which has been shown to be specific for *B. mallei* capable of biosynthesizing LPS (Woods *et al.*, 2002).

The findings of this investigation provide two real-time PCR assays for the specific identification of *B. mallei* and its differentiation from *B. pseudomallei* and closely related *Burkholderia* species. The described assays did not cross-react with any of the bacterial species included in our culture collection, and were capable of detecting *B. mallei* in the lungs, spleen and liver of infected mice beginning at 1 h p.e. This *B. mallei*-specific real-time PCR assay will undoubtedly complement other methodologies used in the clinical laboratory for the rapid identification of this obligate mammalian pathogen.

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