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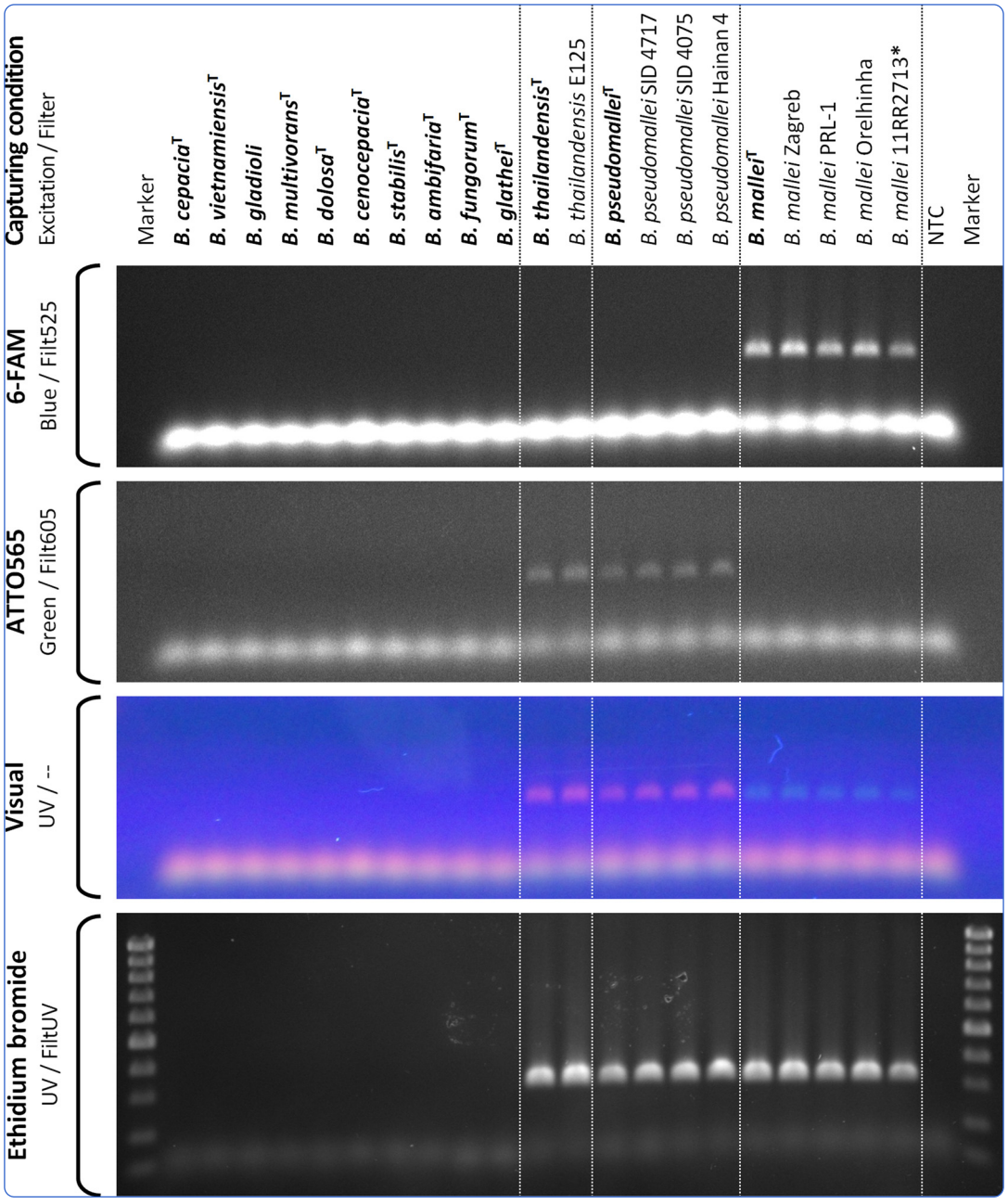

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BRIEF COMMUNICATION Open Access

Clear distinction between *Burkholderia mallei* and *Burkholderia pseudomallei* using fluorescent *mot*B primers

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Abstract

Background: A frame-shift mutation in the flagellum motor gene *mot*B coding for the chemotaxis MotB protein of *Burkholderia mallei* has been utilized to design a conventional duplex PCR assay with fluorescent labelled primers.

Findings: Species specificity was tested with a panel of 13 *Burkholderia* type strains. A total of 41 *B. mallei* field strains, 36 *B. pseudomallei* field strains, and 1 *B. thailandensis* field strain from different geographic regions were tested and correctly identified. Testing of 55 non-*Burkholderia* bacterial species revealed 100% specificity of the assay. The minimum detection limit was 1 pg DNA or 160 GE for *B. mallei* and 130 GE for *B. pseudomallei*, respectively.

Conclusions: This assay enables the clear distinction between *B. mallei* and *B. pseudomallei*/*B. thailandensis*.

Keywords: Duplex PCR, Fluorescent primers, *Burkholderia*

Findings

Despite *Burkholderia mallei*, *B. pseudomallei* and *B. thailandensis* being genetically closely related Gram negative bacteria, they display significant differences in pathogenicity and habitat. *B. mallei*, a facultative intracellular, non-motile, equine pathogen, is the causative agent of glanders, a highly contagious and frequently fatal zoonotic disease of the upper respiratory tract and lungs [1]. The disease has a 95% case fatality rate in untreated humans with septicaemia and a 50% case fatality rate in antibiotic treated individuals [1].

B. pseudomallei, a facultative intracellular, motile bacterium found in contaminated water and soil, is the etiological agent of melioidosis, an infectious disease in man and animal in the tropics [2]. The clinical picture in animals and humans resembles that of glanders in horses. Human infection usually develops after inhalation, ingestion, or cutaneous uptake of the pathogen [2,3]. Melioidosis has a case fatality rate of 39.5%, and untreated septicaemia is fatal in up to 80% of cases [4]. Both *B. mallei* and *B. pseudomallei* are considered potential bioweapons and are listed as category B biothreat agents by the U.S. Centers for Disease Control and Prevention [5]. *B. thailandensis* is generally considered a weakly pathogenic, motile soil bacterium, rarely causing disease in man or animal [6]. Glanders and melioidosis may cause diagnostic problems in endemic regions because of their clinical, morphologic and genetic similarity, and even more so in non-endemic countries, due to the lack of awareness of these diseases. In order to initiate appropriate patient treatment, rapid species identification is necessary, especially in view of the intrinsic resistance of both agents to many commonly used antibiotics and their differing susceptibilities [7,8].

Based on the results from a previous study [9], a frame-shift mutation in the flagellum motor gene *mot*B coding for the chemotaxis MotB protein [GenBank: BMA2861] of *B. mallei* (ATCC 23344) was utilized to design a simple conventional duplex PCR assay with fluorescent labelled primers enabling the distinction between *B. mallei* and *B. pseudomallei*/*B. thailandensis*. Bacterial strains were obtained from the strain collection of the National and OIE Reference Laboratory for Glanders at the Friedrich-Loeffler-Institute in Jena, Germany (Tables 1 and 2). All *Burkholderia* strains were cultured at 37°C on calf blood agar containing 3% (v/v) glycerol.

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Table 1 Panel of *Burkholderia mallei* and *B. pseudomallei* field strains used for validation

Origin	*B. mallei*	*B. pseudomallei*
Africa	-	2
Arabian Peninsula	3	-
Asia	-	1
East Asia	1	4
South Asia	17	5
Southeast Asia	-	13
Europe	3	4
Indonesia	1	-
South America	5	2
Transcontinental Europe/Asia	2	-
Unknown	9	5
Total	**41**	**36**

All other bacteria were grown on standard media and appropriate atmospheric conditions.

Genomic DNA was prepared from culture material using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche, Mannheim, Germany). All DNA samples were quantified using a NanoDrop 1000 spectrophotometer (Fisher Scientific, Schwerte, Germany). The duplex polymerase chain reaction (PCR) was designed using the forward primer MBF04 (5′- CGTCAAGCGGGTGAACCA -3′), the 6-FAM labelled reverse primer MBR04-FAM (5′-6-FAM-GTCGTCCTCGCTCTTTCGC -3′), and the ATTO565 labelled reverse primer MBR10-ATTO565 (5′-ATTO565-GTCCTCGCTCTTCTTCGCG-3′). Primers were designed with the Genious software package (Ver. 6.1), to generate a specific 6-FAM labelled 326 bp DNA fragment for *B. mallei* and an ATTO565 labelled 325 bp DNA fragment for *B. pseudomallei*/*B. thailandensis,* respectively. Labelled

Table 2 Panel of non-Burkholderia strains used for specificity testing

Species	Strain	Species	Strain
Actinobacillus pleuropneumoniae	ATCC 27088	*Legionella pneumophila sub. pneumophila*	DSM 7513
Bacillus atrophaeus	ATCC 9372	*Mannheimia haemolytica*	ATCC 33396
Bacillus brevis	ATCC 8246	*Ochrobactrum anthropi*	CCUG 1047
Bacillus cereus	ATCC 10876	*Oligella urethralis*	DSM 7531
Bacillus megaterium	DSM 90	*Pasteurella multo ssp.multo*	ATCC 43137
Bacillus mycoides	ATCC 6462	*Pasteurella multocida*	DSM 5281
Bacillus subtilis	ATCC 6633	*Proteus mirabilis*	DSM 4479
Bacillus thuringiensis	ATCC 10792	*Pseudomonas aeruginosa*	ATCC 9027
Bartonella henselae	DSM 28221	*Pseudomonas alcaligenes*	ATCC 14909
Bartonella quintana	DSM 21441	*Pseudomonas fluorescens*	ATCC 13525
Bordetella bronchiseptica	ATCC 19395	*Pseudomonas polymyxa*	ATCC 842
Brucella abortus	ATCC 23448	*Pseudomonas putida*	ATCC 12633
Brucella melitensis	ATCC 23456	*Rhodococcus equi*	DSM 20307
Brucella suis	ATCC 23444	*Salmonella enteritidis*	147 (95)
Campylobacter coli	DSM 4689	*Salmonella typhumirium*	9098 (221)
Campylobacter jejuni subsp. jejuni	DSM 4688	*Staphylococcus aureus subsp. aureus*	DSM 6732
Chlamydia abortus	07 DC0059	*Stenotrophomonas maltophilia*	ATCC 13637
Chlamydia pecorum	06 DC0055	*Streptococcus agalactiae*	DSM 6784
Chlamydia psittaci	C1/97	*Streptococcus equi subsp. equi*	ATCC 9528
Clostridium baratii	ATCC 25782	*Streptococcus equi subsp. zooepidemicus*	ATCC 700400
Clostridium botulinum A	NCTC 7272	*Streptococcus equinus*	DSM 20558
Clostridium botulinum B	NCTC 7273	*Streptococcus parauberis*	DSM 6631
Escherichia coli	DSM 30083	*Taylorella equigenitalis*	DSM 10668
Francisella tularensis sub. holarctica	LVS	*Yersinia enterocolitica subsp. enterocolitica*	ATCC 9610
Francisella tularensis sub. tularensis	FSC 237 (SchuS4)	*Yersinia enterocolitica subsp. enterocolitica*	DSM 9499
Haemophilus influenzae	ATCC 9006	*Yersinia enterocolitica subsp. palearctica*	DSM 13030
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104	*Yersinia pseudotuberculosis*	IP32953
Lactobacillus ruminis	DSM 20403		

primers were obtained from Microsynth (Balgach, Switzerland), the unlabelled primer from Jena Bioscience (Jena, Germany). PCR was conducted in a 20 μL reaction containing 0.3 μM of the primers (MBF04, MBR04-FAM, and MBR10-ATTO565), 1 × 5-Prime HotMaster-Mix (VWR, Darmstadt, Germany), 2.5% DMSO and 10 ng template (total DNA). The PCR was performed in a Mastercycler pro S™ (Eppendorf, Germany) under the following conditions: initial denaturation at 95°C for 1 min; 40 cycles at 95°C for 10 s, 63°C for 15 s, 70°C for 30 s, and the final extension at 70°C for 5 min. 13.3 μL PCR reaction mixed with 2.7 μL 6 × Loading Dye (Fermentas, Schwerte, Germany) were analysed by electrophoresis on a 1.25% agarose gel (wt/vol) at 9 V/cm for 40 min. Images were captured after an exposure period of 30 s for each LED/filter set using the G-Box EF2 Gel Documentation System (Syngene Europe, Cambridge, UK): Blue-LED/Filt525 and Green-LED/Filt605 for the visualisation of 6-FAM and ATTO565 labelled PCR products, respectively. For optional ethidium bromide imaging (302 nm UV illuminator/FiltUV), the gel was stained after capturing the 6-FAM/ATTO565 images. Fragment sizes (326/327 bp) and correct labelling (6-FAM/ATTO565) of the amplicons were confirmed by means of capillary electrophoresis using a Genetic Analyzer 3130 with a G5 filter set (Applied Biosystems/Hitachi, Darmstadt, Germany). Species specificity was tested with a panel of 13 *Burkholderia* type strains. Additionally, a total of 41 *B. mallei* field strains from equines, 36 *B. pseudomallei* field strains from human and environmental origin, and one *B. thailandensis* field strain, all from different geographic regions were tested and correctly identified (Table 1). Testing of 55 non-*Burkholderia* bacterial species revealed 100% specificity of the assay (Table 2). The minimum detection limit was 1 pg DNA or 160 genome equivalents (GE) for *B. mallei* and 130 GE for *B. pseudomallei*, respectively. In order to compare the sensitivity of our assay with other assays used by the National and OIE Reference Laboratory for Glanders, several clinical *B. mallei* samples were tested by a conventional *fli*P PCR [10] and a real time PCR assay targeting *fli*C [11]. Despite the lower sensitivity we determined for our assay, it revealed comparable sensitivity to the conventional *fli*P PCR and a higher sensitivity than the real time *fli*C assay in the tested clinical samples (Additional file 1).

Fluorescent primers are widely used in real time PCR technology and several highly sophisticated and elegant PCR assays have been developed for the identification and differentiation of *B. mallei* and *B. pseudomallei* and other *Burkholderia* species in the past few years [12]. This study describes the design of a simple conventional duplex PCR with fluorescent labelled primers for amplifying species-specific amplicons of *B. mallei* and *B. pseudomallei*/*B. thailandensis*, respectively. These closely related species can cause considerable problems during the identification process in the laboratory as colony characteristics and routine biochemical tests are not sufficiently discriminative for species identification. The benefit of this assay is not only the unambiguous identification of *B. mallei* and the closely related species *B. pseudomallei* and *B. thailandensis* by fluorescence image capturing but also the possibility of detecting the *B. mallei/pseudomallei/thailandensis* complex on a standard ethidium bromide stained agarose gel.

Additional file

Additional file 1: Comparison of the *mot*B PCR assay to the conventional *fli*P and real time *fli*C PCR assays in clinical samples (*Burkholderia* type strains ATCC 23343 T, ATCC 23344T).

Abbreviations

ATCC: American type culture collection; CCUG: Culture collection university of Göteborg; DSM: Deutsche Sammlung von Mikroorganismen; FAM: Fluorescein; FSC: *Francisella* strain collection, Sweden; GE: Genome equivalent; LED: Light-emitting diode; NCTC: National collection of type cultures.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GS and ME designed the study; LDS drafted and wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Dvorak GD, Spickler AR. Glanders. J Am Vet Med Assoc. 2008;233:570–7.
2. Cheng AC, Dance DA, Currie BJ. Bioterrorism Glanders and Melioidosis. Euro Surveill. 2005;10:3.
3. Wiersinga WJ, Currie BJ, Peacock SJ. Melioidosis. N Engl J Med. 2012;367:1035–44.
4. Zysk G, Splettstösser WD, Neubauer H. A review on melioidosis with special respect on molecular and immunological diagnostic techniques. Clin Lab. 2000;46:119–30.
5. Rotz LD, Khan AS, Lillibridge SR, Ostroff SM, Hughes JM. Public health assessment of potential biological terrorism agents. Emerg Infect Dis. 2002;8:225–30.
6. Brett PJ, DeShazer D, Woods DE. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int J Syst Bacteriol. 1998;48:317–20.
7. Thibault FM, Hernandez E, Vidal D, Girardet M, Cavallo JD. Antibiotic susceptibility of 65 isolates of *Burkholderia pseudomallei* and *Burkholderia mallei* to 35 antimicrobial agents. J Antimicrob Chemother. 2004;54:8.

8. Gilad J. *Burkholderia mallei* and *Burkholderia pseudomallei*: the causative micro-organisms of glanders and melioidosis. Recent Pat Antiinfect Drug Discov. 2007;2:233–41.
9. Schmoock G, Ehricht R, Melzer F, Rassbach A, Scholz HC, Neubauer H, et al. DNA microarray-based detection and identification of *Burkholderia mallei*, *Burkholderia pseudomallei* and Burkholderia spp. Mol Cell Probes. 2009;23:178–87.
10. Scholz HC, Joseph M, Tomaso H, Al Dahouk S, Witte A, Kinne J, et al. Detection of the reemerging agent *Burkholderia mallei* in a recent outbreak of glanders in the United Arab Emirates by a newly developed *fli*P-based polymerase chain reaction assay. Diagn Microbiol Infect Dis. 2006;54:241–7.
11. Tomaso H, Scholz HC, Al Dahouk S, Pitt TL, Treu TM, Neubauer H. Development of 5′ nuclease real-time PCR assays for the rapid identification of the *Burkholderia mallei//Burkholderia pseudomallei* complex. Diagn Mol Pathol. 2004;13:247–53.
12. Lowe W, March JK, Bunnell AJ, O'Neill KL, Robison RA. PCR-based methodologies used to detect and differentiate the *Burkholderia pseudomallei* complex: *B. pseudomallei*, *B. mallei*, and *B. thailandensis*. Curr Issues Mol Biol. 2013;16:23–54.