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Identification and Discrimination of *Burkholderia pseudomallei*, *B. mallei*, and *B. thailandensis* by Real-Time PCR Targeting Type III Secretion System Genes

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***Burkholderia pseudomallei* and *B. mallei* are two highly pathogenic bacteria, responsible for melioidosis and glanders, respectively. The two are closely related and can also be mistaken for *B. thailandensis*, a nonpathogenic species. To improve their differential identification, we describe a hydrolysis probe-based real-time PCR method using the uneven distribution of type III secretion system genes among these three species.**

Burkholderia pseudomallei is the gram-negative motile bacterium responsible for melioidosis. This saprophyte inhabitant of telluric environments is mainly encountered in Southeast Asia and northern Australia but is sporadically isolated in subtropical and temperate countries (18). Melioidosis is a life-threatening disease that is mainly acquired though skin inoculation or pulmonary contamination, although other routes have been documented. *B. mallei*, a gram-negative nonmotile bacterium, is the causative agent of glanders. This disease mainly affects horses; humans can be infected after prolonged and close contact with these animals (11). *B. pseudomallei* and *B. mallei* are phylogenetically very similar and have nearly identical 16S ribosomal DNA sequences. They have been defined as separate species essentially due to their epidemiological and sanitary features (21). Both species are highly pathogenic and are listed in biological risk class III. Moreover, due to their high virulence by the respiratory route, both are considered potential bioterrorism agents, classified as such in list B by the Centers for Disease Control and Prevention (Atlanta, Ga.) (13). *B. thailandensis* is a gram-negative, motile saprophyte bacterium. This organism is nonpathogenic for humans and animals but displays phenotypic characteristics that make it appear similar to *B. pseudomallei* by routine diagnosis tests. Its main specific character is the ability to assimilate L-arabinose. Before being regarded as a definite species (5), *B. thailandensis* isolates were previously described as arabinose-positive, nonpathogenic variants of *B. pseudomallei*, as opposed to the arabinose-negative, pathogenic sensu stricto *B. pseudomallei* strains (14).

As pathogenic *Burkholderia* isolates are quite infrequent in clinical practice outside of the areas of endemicity, their identification could be missed, even when automated systems are used (9). PCR methods have been proposed, but these suffer from a lack of specificity (7, 15).

The type III secretion system (TTS) is a toxin delivery mechanism that allows pathogenic bacteria to inject toxic substances into the cytoplasm of the host's cells. This "toxin gun" was first described for *Salmonella* spp. and *Shigella flexneri* (3, 6) but is now documented in numerous animal- and plant-pathogenic bacteria (8, 22). This mechanism relies on a multiprotein assembly showing strong similarities with the flagella structure (1, 4).

Winstanley et al. (19) reported the first TTS gene cluster, TTS1, in *B. pseudomallei*. The presence of TTS1 has been cor-

TABLE 1. List of PCR primers and hydrolysis probes

Target gene	Primer	Sequence[a] (5′–3′)	Reference or source	PCR amplification			
				B. pseudomallei	*B. mallei*	*B. thailandensis*	Other species
orf11	PM122	ATCGCCAAATGCCGGGTTTC	12	+	−	−	−
	orf11R	CAAATGGCCATCGTGATGTTC	This study				
	orf11pro	FAM-TCGGCGAACGCGATTTGATCGTTC-TAMRA	This study				
orf13	orf13f	CACCGGCAGTGATGAGCCAC	This study	+	+	−	−
	orf13r	ATGCTCCGGCCTGACAAACG	This study				
	orf13pro	FAM-ACGCCCGTCGAAGCCCGAATC-TAMRA	This study				
BpSCU2	SCU2F	CTCGAGCTCGTGAAGATGAT	This study	+	+	+	−
	SCU2R	ACGCGTGCGATCTTGTAATC	This study				
	SCU2pro	FAM-ATGCCACGCACGCGAGCACGA-TAMRA	This study				

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

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TABLE 2. List of strains used in this study

Species	Strain	Source
B. andropogonis	ATCC 2306	American Type Culture Collection (Manassas, Va.)
B. cepacia	ATCC 25416	
B. mallei	ATCC 10399	
B. mallei	ATCC 23344	
B. pseudomallei	ATCC 11668	
B. pseudomallei	ATCC 15682	
B. pseudomallei	ATCC 23343	
B. thailandensis	ATCC 700388	
B. mallei	CIP 52.236	Collection de l'Institut Pasteur (Paris, France)
B. mallei	CIP 64.12	
B. mallei	CIP A.187	
B. mallei	CIP A.198	
B. mallei	CIP A.199	
B. mallei	CIP A.200	
B. pseudomallei	CIP 52.238	
B. pseudomallei	CIP 52.239	
B. pseudomallei	CIP 55.135	
B. pseudomallei	CIP 60.67	
B. pseudomallei	CIP 62.27	
B. pseudomallei	CIP 62.28	
B. pseudomallei	CIP 68.2	
B. pseudomallei	CIP 68.3	
B. pseudomallei	CIP A.202	
B. pseudomallei	CIP A.203	
P. aeruginosa	CIP 100720	
B. norimbergensis	DSMZ 11628	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany)
B. caledonica	LMG 19076	Belgian Coordinated Collection of Microorganisms (Ghent, Belgium)
B. caribensis	LMG 18531	
B. caryophylli	LMG 2155	
B. cenocepacia	LMG 12614	
B. dolosa	LMG 18941	
B. fungorum	LMG 16225	
B. gladioli	LMG 2216	
B. glathei	LMG 14190	
B. glumae	LMG 2196	
B. multivorans	LMG 13010	
B. phenazinium	LMG 2247	
B. plantarii	LMG 10907	
B. pyrrocinia	LMG 14191	
B. stabilis	LMG 14294	
B. vietnamiensis	LMG 10929	
B. mallei	NC 00120	National Collection of Type Cultures (London, United Kingdom)
B. mallei	NC 03708	
B. mallei	NC 03709	
B. mallei	NC 10229	
B. mallei	NC 10230	
B. mallei	NC 10247	
B. mallei	NC 10248	
B. mallei	NC 10260	
B. pseudomallei	NC 01688	
B. pseudomallei	NC 04846	
B. pseudomallei	NC 06700	
B. pseudomallei	NC 07383	
B. pseudomallei	NC 07431	
B. pseudomallei	NC 08016	
B. pseudomallei	NC 08707	
B. pseudomallei	NC 08708	
B. pseudomallei	NC 10274	
B. pseudomallei	NC 10276	
B. pseudomallei	E 090	Public Health Laboratory Service (London, United Kingdom; strains kindly provided by T. Pitt)
B. pseudomallei	E 222	
B. pseudomallei	SID 3783	
B. pseudomallei	SID 3871	
B. pseudomallei	SID 4717	
B. pseudomallei	SID 4718	
B. pseudomallei	SID 4935	
B. pseudomallei	SID 5278	
B. thailandensis	E 027	
B. thailandensis	E 082	
B. pseudomallei	56.91	Institut Pasteur (Paris, France; strains kindly provided by A. Dodin)
B. pseudomallei	70061	
B. pseudomallei	77804	
B. pseudomallei	A120	
B. pseudomallei	Ducruet	
B. pseudomallei	NT16	
B. pseudomallei	PA.1195	
B. pseudomallei	W5	
B. graminis	AUS 28	Laboratoire d'Ecologie Microbienne (Université Claude Bernard, Villeurbanne, France; strains kindly provided by B. Cournoyer)
B. graminis	AUS 35	
B. graminis	C3A1M	
B. graminis	C4D1M	
B. graminis	C5A1M	
B. cepacia	001/97	Centre de Recherches du Service de Santé des Armées (Grenoble, France)
B. cepacia	002/97	
B. pseudomallei	008/93	
B. pseudomallei	013/97	
B. pseudomallei	041/97	
B. pseudomallei	042/97	
B. pseudomallei	043/97	
B. pseudomallei	047/98	
B. pseudomallei	050/98	
B. pseudomallei	062/00	
B. pseudomallei	067/00	
B. pseudomallei	089/01	
B. pseudomallei	164/03	
B. pseudomallei	9	Defense Medical and Environmental Research Institute (Singapore; DNAs kindly provided by May Ann Lee)
B. pseudomallei	11	
B. pseudomallei	22	
B. pseudomallei	59	
B. pseudomallei	15-10	
B. pseudomallei	5/96	
B. pseudomallei	15/96	
B. pseudomallei	497/96	

related with an Ara$^-$ phenotype (20) and with pathogenicity (16, 17). An in silico study (12) demonstrated the presence of a partial TTS1 analog in *B. mallei*. A second type III secretion system (TTS2) has been demonstrated for *B. pseudomallei*, *B. mallei*, and *B. thailandensis* (12); the role of these genes has not yet been elucidated.

The present study shows that the uneven distribution of TTS genes among *B. pseudomallei*, *B. mallei*, and *B. thailandensis* provides a means for distinguishing these three species by PCR.

Primer and probe design. Real-time PCR assays using hydrolysis probes were designed for three genetic markers: *orf11* and *orf13* from TTS1 and *BpSCU2* from TTS2 (Table 1). The sequences of these open reading frames were obtained from the recently completed *B. pseudomallei* K96243 genome sequence. These sequence data were produced by the *B. pseudomallei* Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bps/; they are consistent with the TTS1 sequence deposited by Winstanley et al. under the GenBank accession number AF074878. Sequence specificity was checked by (i) BLAST searches for nearly exact matches via the site http://www.ncbi.nlm.nih.gov/BLAST/ and (ii) analysis of the genome sequences of *B. mallei*

(available at The Institute for Genomic Research website http://www.tigr.org) and of *B. cenocepacia* (available at the Sanger Institute website http://www.sanger.ac.uk/Projects/B_cenocepacia) performed using the BLAST facilities provided at these websites. The three markers studied exhibited identities only with sequences from *B. pseudomallei*, *B. mallei*, or *B. thailandensis.* No significant similarities with sequences from other bacteria, even from beta-proteobacteria, such as members of the *B. cepacia* complex or *Ralstonia* species, were found. Primers and probes were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, Calif.).

PCR. Real-time PCR assays were conducted on a LightCycler apparatus (Roche Applied Science, Penzberg, Gerdany), using FastStart hybridization probe master mixture (Roche) by following the manufacturer's instructions. Primers and probes were obtained from MWG Biotech (Courtaboeuf, France) and were used at final concentrations of 0.5 and 0.2 μM, respectively. Final $MgCl_2$ concentrations were adjusted to 3, 2, and 4 mM for detection of *orf11*, *orf13*, and *BpSCU2*, respectively. Sample volume was 5 μl per assay. Thermal cycling conditions were 5 min at 95°C, followed by 45 cycles of 10 s at 95°C and 45 s at 60°C.

Bacterial strains. The following species where studied (Table 2; numbers of strains are in parentheses): *B. pseudomallei* (58), *B. mallei* (16), *B. thailandensis* (3), *B. andropogonis* (1), *B. caledonica* (1), *B. caribensis* (1), *B. caryophylli* (1), *B. cepacia* (3), *B. cenocepacia* (1), *B. dolosa* (1), *B. fungorum* (1), *B. gladioli* (1), *B. glathei* (1), *B. glumae* (1), *B. graminis* (5), *B. multivorans* (1), *B. norimbergensis* (1), *B. phenazinium* (1), *B. plantarii* (1), *B. pyrrocinia* (1), *B. stabilis* (1), *B. vietnamiensis* (1), *Pseudomonas aeruginosa* (1). Species identification was confirmed by routine phenotypic characterization including Gram staining, motility tests, and biochemical profiles on API 20 NE tests (BioMérieux, Marcy l'Etoile, France) (2).

Samples. PCR was performed, with similar qualitative results, on (i) DNA purified by phenol-chloroform extraction by standard procedures (10) and (ii) water-washed or paraformaldehyde-fixed whole bacteria from broth or agar cultures.

Differential identification. *BpSCU2* was amplified from *B. pseudomallei*, *B. mallei*, and *B. thailandensis*. Only the two highly pathogenic species, *B. pseudomallei* and *B. mallei*, appeared positive for *orf13*. Only *B. pseudomallei* generated the *orf11* amplicon. No amplification of the three targets occurred with any of the other species tested (Table 1). These results are consistent with those of BLAST sequences analysis.

Quantification. The detection limit was 5 fg of *B. pseudomallei* DNA/μl. When determined on serial 10-fold dilutions of genomic DNA, the quantification was linear between 5 ng/μl and 0.05 pg/μl.

Probe specificity. No interference was observed when amplifying *orf11* from *B. pseudomallei* 23343 DNA diluted in *B. mallei* 23344 DNA to a ratio of 1:1,000.

Standards. Plasmid standards were constructed by cloning fragments amplified from *B. pseudomallei* 23343 DNA in pCR2.1 TOPO plasmids (Invitrogen, Carlsbad, Calif.) by following the manufacturer's instructions. Purified plasmids were linearized with BamHI endonuclease before use in PCR.

In conclusion, this work provides a means for diagnosis and discrimination between three closely related species, *B. pseudomallei*, *B. mallei*, and *B. thailandensis*, by use of type III secretion system genes. Among the genes studied, *orf13* can be regarded as specific for the two highly pathogenic species and *orf11* is a specific marker for *B. pseudomallei. BpSCU2* is shared by the three species, but, when it is the only marker detected, it provides a means for identification of *B. thailandensis* in addition to arabinose assimilation.

As hydrolysis probe technology uses standard cycling parameters regardless of the length and sequence of the amplicon, the different markers can be detected during the same LightCycler run. Plasmid standards can be used as positive controls more safely than bacterial cultures or genomic DNA.

This method affords considerable improvements in the specificity and rapidity of the diagnosis of these pathogens and allows rapid discrimination from opportunistic pathogens, such as members of the *B. cepacia* complex, that routine diagnostic laboratories are more likely to encounter. Moreover, due to the high risks associated with handling of *B. pseudomallei* and *B. mallei*, the molecular technique described here can be used by level A laboratories for identification of these potential bioterrorism agents with minimal culture steps.

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