Development and Evaluation of a Real-Time PCR Assay Targeting the Type III Secretion System of *Burkholderia pseudomallei*

Ryan T. Novak,[1] Mindy B. Glass,[1]* Jay E. Gee,[1] Daniel Gal,[2] Mark J. Mayo,[2]
Bart J. Currie,[2] and Patricia P. Wilkins[1]

*Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases,
Centers for Disease Control and Prevention, Atlanta, Georgia 30333[1]; and Menzies School of Health Research,
Charles Darwin University, Northern Territory Clinical School, Flinders University,
Royal Darwin Hospital, Darwin, Northern Territory, Australia[2]*

Here we report on the development of a discriminatory real-time assay for the rapid identification of *Burkholderia pseudomallei* isolates and the evaluation of this assay for sensitivity against related species and detection in spiked human blood samples. The assay targets a 115-base-pair region within *orf2* of the *B. pseudomallei* type III secretion system gene cluster and distinguishes *B. pseudomallei* from other microbial species. Assay performance was evaluated with 224 geographically, temporally, and clinically diverse *B. pseudomallei* isolates from the Centers for Disease Control and Prevention strain collection. This represents the first real-time PCR for rapid and sensitive identification of *B. pseudomallei* that has been tested for cross-reactivity with 23 *Burkholderia mallei*, 5 *Burkholderia thailandensis*, and 35 *Burkholderia* and 76 non-*Burkholderia* organisms which have historically presented diagnostic challenges. The assay performed with 100% specificity. The limit of detection was found to be 76 femtograms of DNA (equivalent to 5.2×10^3 genome equivalents per ml) in a single PCR. In spiked human blood, the assay could detect as few as 8.4×10^3 CFU per ml. This rapid assay is a valuable tool for identification of *B. pseudomallei* and may improve diagnosis in regions endemic for melioidosis.

Melioidosis, a disease caused by infection with *Burkholderia pseudomallei*, is a significant public health burden in Southeast Asia and northern Australia, where in some regions it is the most common cause of fatal community-acquired bacteremic pneumonia (6, 45). The ecological distribution of *B. pseudomallei* is mostly limited to the tropical and subtropical areas between 20° north and south latitudes. While occasional cases of melioidosis occur beyond these regions, outside the tropics they have usually been associated with imported animals or have occurred in travelers returning from these regions (6, 7, 12). Clinical manifestations of melioidosis range from asymptomatic infection to focal infections in skin, muscle, bones, joints, or internal organs to pneumonia, which can be chronic or fulminant with rapidly fatal septicemia, where death can occur within 48 h of symptom onset (6, 8, 45). *B. pseudomallei* infection can resemble a number of other infections including typhoid, tuberculosis, and malaria (45). High infectivity, severity of disease, and environmental persistence have resulted in *B. pseudomallei* being classified as a category B select agent by the Centers for Disease Control and Prevention (CDC) (31).

Culture remains the current "gold standard" for diagnosis of melioidosis and identification of *B. pseudomallei* from a clinical specimen (43, 48, 50). However, definitive identification relies on a battery of traditional microbiological tests that can require up to a week to complete. Furthermore, some biochemical identification methods for culture isolates are problematic, especially rapid biochemical assays which have been shown to misidentify *B. pseudomallei* as other bacteria such as *Pseudomonas* spp., *Burkholderia vietnamiensis*, *Stenotrophomonas maltophilia*, and in particular *Chromobacterium violaceum*, which can cause an infection with similar symptoms to melioidosis (14, 19, 23). Available serologic assays (dot immunoassay, indirect hemagglutination assay, enzyme-linked immunosorbent assay, and immunochromatograpic test) are not particularly useful in regions endemic for melioidosis because past exposure to *B. pseudomallei* may result in seroconversion (6, 27, 45). Rapid bacterial detection from clinical samples using latex agglutination or direct immunofluorescence has been successful in Thailand, but these assays are not commercially available (42, 49). Compounding the need for rapid diagnosis is the lack of a human vaccine and the intrinsic resistance of *B. pseudomallei* to many of the affordable antibiotic treatments for general sepsis in areas endemic for the disease (7, 20, 45). In addition to accelerating appropriate therapeutic intervention, improved rapid diagnostic tests could potentially prevent unnecessary laboratory-acquired infection due to overhandling of clinical specimens containing *B. pseudomallei* and would facilitate surveillance prior to and following deliberate release (3, 5, 7, 31).

The complete sequence of the *B. pseudomallei* genome reveals several gene targets for the identification of this species (e.g., 16S rRNA genes, *fliC*, *rpsU*, and type III secretion [TTS] gene cluster) (18, 25, 26, 36, 38, 39). This approach to PCR target identification and primer and probe design and improved methods for fluorescence labeling of DNA allow for the in silico design and evaluation of real-time PCR assays specific

* Corresponding author. Mailing address: Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC, MS-G34, 1600 Clifton Rd., N.E., Atlanta, GA 30333. Phone: (404) 639-4055. Fax: (404) 639-3023. E-mail: mglass@cdc.gov.

TABLE 1. Microorganisms ($n = 139$) used to test the specificity of the TTS1 real-time PCR assay

Isolate category	Species, genomovar, serovar, serogroup, and/or strain[a]	No. tested
Genetic relatives ($n = 78$)	*Burkholderia cepacia* genomovar I/III, *B. multivorans*, *B. stabilis*, *B. gladioli*, *B. vietnamiensis*[T], *B. cocovenenans*, *Burkholderia* sp. strain Oklahoma	35
	Burkholderia mallei	23
	Burkholderia thailandensis	5
	Pandoraea apista, *P. norimbergensis*, *P. pnomenusa*[T], *P. sputorum*	8
	Ralstonia paucula, *R. pickettii Ralstonia* sp.	7
Differential diagnoses ($n = 11$)	*Acinetobacter baumannii*[T]	1
	Mycobacterium tuberculosis[T]	1
	Plasmodium falciparum[T], *P. malariae*[T], *P. ovale*[T], *P. vivax*[T]	4
	Staphylococcus aureus	1
	Vibrio cholerae	2
	Yersinia enterocolitica O:9, *Y. enterocolitica*	2
Potential misidentifications ($n = 6$)	*Alcaligenes faecalis*	1
	Chromobacterium violaceum	3
	Stenotrophomonas maltophilia	2
Select agents ($n = 6$)	*Bacillus anthracis*	2
	Brucella abortus, *B. melitensis*, *B. suis*	3
	Francisella tularensis subsp. *holarctica* strain LVS	1
Potential sample contaminants ($n = 38$)	*Achromobacter xylosoxidans*	1
	Aeromonas hydrophila[T]	1
	Afipia broomeae	1
	Bartonella clarridgeiae[T], *B. henselae*[T]	2
	Bacillus cereus	3
	Brucella canis	1
	Candida albicans[T]	1
	Enterobacter aerogenes	1
	Escherichia coli serotype O157:H7, *E. coli*[T]	2
	Haemophilus influenzae	2
	Klebsiella pneumoniae	1
	Legionella pneumophila[T]	1
	Ochrobactrum anthropi 5D	3
	Proteus mirabilis[T], *P. vulgaris*	2
	Pseudomonas aeruginosa[T], *P. mendocina*, *P. putida*, *P. stutzeri*	9
	Psychrobacter phenylpyruvicus[T]	1
	Rothia dentocariosa	1
	Salmonella enterica serovar Urbana, *Salmonella* spp.	3
	Sphingobacterium multivorum	1
	Streptococcus pneumoniae	1

[a] Superscript T indicates the type strain.

for a given species (15, 24, 29, 30). The disadvantage of a purely theoretical approach is choosing gene targets based on one strain, which may not be representative of the global *B. pseudomallei* population, resulting in false-negative results. Thus for this study we chose a target based on not only its theoretical specificity but also its apparent ubiquitous population distribution (35). Previously, a 548-bp region of the *B. pseudomallei* type III secretion system (TTS1) gene cluster encompassing part of open reading frame 2 (*orf2*) was found to be present in *B. pseudomallei* and not in the related *Burkholderia mallei* or *Burkholderia thailandensis* (28, 46, 47). This open reading frame was later found to be present in all of a large number of northern Australian *B. pseudomallei* strains (35). We report here the design and evaluation of a fluorescence-labeled-probe-based real-time PCR assay targeting *orf2* of the TTS1 of *B. pseudomallei* (TTS1 real-time PCR). The sensitivity, specificity, low limit of detection, and lack of interaction with human DNA make this assay a good candidate for diagnostic clinical laboratory evaluation.

MATERIALS AND METHODS

Collection and preparation of bacterial strains. Bacterial strains were obtained from CDC culture collections housed in the Meningitis and Special Pathogens Branch. They included 224 *B. pseudomallei* strains representing temporal (1949 to 2004), geographic (Americas, Asia, Europe, Africa, and Oceania), and origin diversity (human, environmental, animal). The geographic regions covered by this collection represent the locale of isolation and not necessary where infection occurred. Isolates from regions not traditionally considered endemic for melioidosis such as North America and Europe represent imported cases. In addition, 23 *B. mallei* strains, 5 *B. thailandensis* strains, and 111 non-*B. pseudomallei* strains were evaluated for cross-reactivity (Table 1). All strains were stored at −70°C in defibrinated rabbit blood until they were tested. Identification of strains was carried out with a standard battery of biochemical tests (44). Culture isolates were not available from six of the diagnostically challenging organisms (*Mycobacterium tuberculosis*, *Francisella tularensis*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*). For these pathogens DNA was obtained from subject matter expert laboratories and tested directly.

DNA preparation. Isolates were cultured on standard media at 37°C overnight. For testing assay specificity whole-cell DNA extractions were prepared from the cultures by heat lysis as previously described by Hoffmaster et al. (17). The assay sensitivity was evaluated using purified *B. pseudomallei* genomic DNA extracted

with the DNeasy tissue kit (QIAGEN, Valencia, CA) using the manufacturer's instructions for gram-negative bacteria. For spiked human blood samples, DNA was isolated from a 200-μl sample using a modified protocol for the QIAmp DNA blood kit (QIAGEN), substituting proteinase K for protease and buffer ATL for AL and using an elution volume of 200 μl. The procedure was modified to add a 10-min 70°C step to ensure lysis of bacterial cells. The DNA concentrations were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the numbers of genome equivalents (GE) were calculated using the 7.25-Mbp genome (68% G+C) of *B. pseudomallei* K96243 (18).

Real-time PCR assay. Primer pair and probe were designed to target the type III secretion system gene cluster of *B. pseudomallei* (GenBank accession no. AF074878) using Primer Express 2.0 (Applied Biosystems, Inc., Foster City, CA), BpTT4176F (5′-CGTCTCTATACTGTCGAGCAATCG-3′), and BpTT4290R (5′-CGTGCACACCGGTCAGTATC-3′). The primer pair was designed to generate a 115-bp PCR product. The fluorogenic probe BpTT4208P (5′-CCGGAATCTGGATCACCACCACTTTCC-3′) was synthesized with a 6-carboxyfluorescein reporter molecule attached at the 5′ end and a Black Hole Quencher 1 on the 3′ end. The primers and probe were synthesized in the Biotechnology Core Facility at the CDC.

Each TTS1 real-time PCR mixture contained a 2-μl aliquot of extracted DNA in 23 μl of a PCR master mixture containing 1× LightCycler Faststart DNA master hybridization probe reaction mixture (Roche Applied Sciences, Indianapolis, IN), 5 mM MgCl$_2$, 400 nM of each primer, and 200 nM of probe. Amplification and detection were performed with a SmartCycler (Cepheid, Sunnyvale, CA) using the standard settings. The SmartCycler thermal cycling conditions were 95°C for 8 min, followed by 45 cycles of 15 s at 95°C and 15 s at 59°C. The cycle threshold values represent the calculated cycles at which fluorescence from the cleaved probe exceeds a fixed threshold, as calculated by the instrument for each reaction. No-template (PCR grade water) and positive (*B. pseudomallei* CDC2000032028) controls were included in each run to rule out the possibility of contamination or amplification failure. The sensitivity and specificity of the real-time PCR assay were evaluated by testing 1 ng of extracted DNA.

The lower limit of detection (LLOD), linear dynamic range (LDR), and assay variance for the TTS1 real-time PCR were determined using purified *B. pseudomallei* (CDC2000032028) DNA (13). For LDR and variance analysis, five replicates from a 10-fold serial dilution (10^8 to 10^3 GE/ml) of purified DNA in elution buffer (QIAGEN, Valencia, CA) were assayed in a single run. To determine LLOD, an additional five replicates from a 10-fold serial dilution (10^2 to 10^{-2} GE/ml) of purified DNA were added. Eight separate replicate experiments were conducted using aliquots of DNA frozen at −20°C. These data were compared to those obtained from DNA extractions of six spiked human blood samples (Lampire Biological, Coopersburg, PA, and CDC Blood Services Group, Atlanta, GA). Previous studies have highlighted the need for preassay enrichment of clinical samples to achieve DNA concentrations in the range of real-time detection (38, 39). Therefore, we designed an evaluation of the assay for detecting *B. pseudomallei* cells in human blood. An aliquot of the uninoculated whole human blood (with EDTA as a non-PCR-inhibiting anticoagulant) was plated prior to each experiment to check for sterility. Colonies from overnight cultures of *B. pseudomallei* (CDC2000032028) were suspended in phosphate-buffered saline (PBS) at a concentration equivalent to 10^9 cells per ml based on McFarland turbidimetric standards. Serial 10-fold dilutions (10^8 to 10^{-2} CFU/ml) were made from the solution in blood and PBS. Aliquots from each series of dilutions were used for spread plates in triplicate, and the average CFU/ml was calculated. Four replicates of each series were assayed.

Data and statistical analysis. The LLODs were determined using a model of nonlinear regression (PriProbit version 1.63). The LDR and the coefficient-of-variation calculations for the assay were carried out with Excel (Microsoft). The PCR efficiency (E) of the primer pair and probe was calculated using the equation $E = 10^{-1/slope} - 1$ (40). An E of 1.0 indicates that amplicon quantity is duplicated every cycle.

RESULTS

Sensitivity and specificity. The assay successfully amplified DNA from all 224 *B. pseudomallei* strains in the CDC culture collection. The cycle threshold values ranged from 19 to 27 for the whole-cell extracts. Using a nonlinear-regression model, the LLOD from purified *B. pseudomallei* (CDC2000032028) DNA was 76 fg/PCR (equivalent to 5.2×10^3 GE/ml) (Fig. 1A), and the LDR was 3.3×10^4 GE/ml to 3.3×10^8 GE/ml
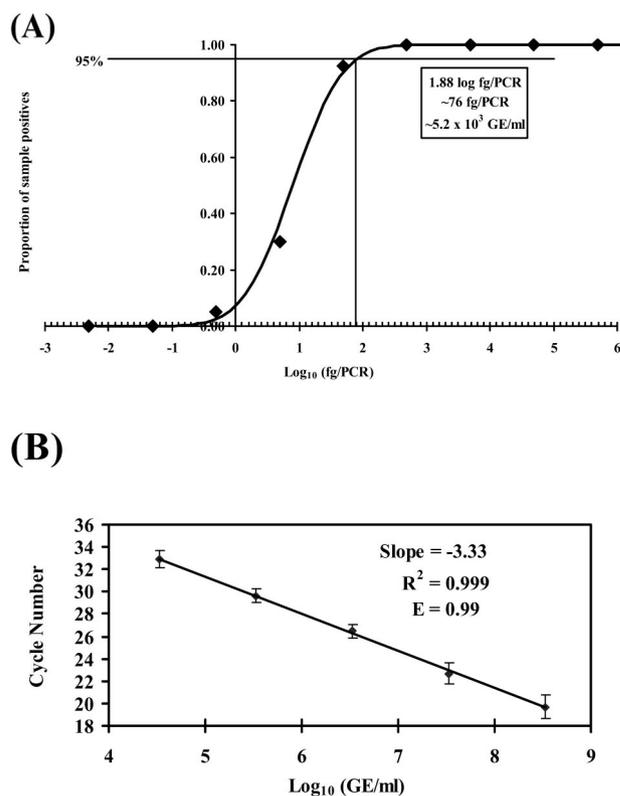

FIG. 1. Limits of detection and linear dynamic range of TTS1 PCR using *Burkholderia pseudomallei* DNA extracts. (A) Probit nonlinear regression analysis of the real-time PCR assay's detection of the TTS1 gene target. (B) Dynamic range analysis of the real-time PCR assay's detection of the TTS1 gene target. The regression line represents data in the linear range. Error bars equal two standard deviations of the aggregate mean. The slope, correlation coefficient (R^2), and PCR efficiency (E) are displayed.

(Fig. 1B). Intra-assay variance was estimated in the LDR to be between 0.39 and 1.64%, while the interassay variability was between 1.00 and 1.76%.

The specificity of the designed primers and probe was assessed first in silico by comparing the sequences and the gene product using BLAST (2). There were no sequences in the database producing alignments with significant expectation values or sharing regions of significant similarity to the primers or probe.

All non-*B. pseudomallei* isolates were negative (Table 1). These included genetic relatives (e.g., *Burkholderia mallei*, *B. thailandensis*, and *Ralstonia* spp.), biothreat agents (e.g., *Bacillus anthracis*, *Brucella* spp., and *Francisella tularensis*), and pathogens that are part of the differential diagnoses for melioidosis (e.g., *Staphylococcus aureus*, *Plasmodium* spp., *Salmonella* spp., and *Mycobacterium tuberculosis*). Organisms that cause frequent misidentifications using rapid biochemical assays (e.g., *Pseudomonas* spp., *Burkholderia vietnamiensis*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, and *Chromobacterium violaceum*) were negative by the TTS1 real-time assay.

Assay of spiked blood samples. Human DNA was nonreactive by the TTS1 real-time PCR. Five separate experiments were performed using spiked blood samples from five different donors with heterogeneous blood types and travel histories.
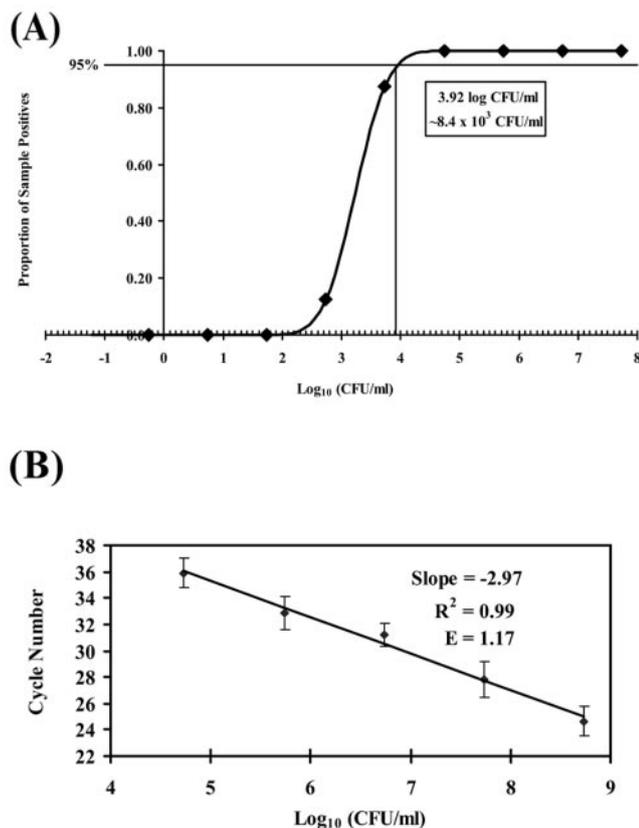
FIG. 2. Limits of detection and linear dynamic range of TTS1 PCR using *Burkholderia pseudomallei*-spiked human blood. (A) Probit nonlinear regression analysis of the real-time PCR assay's detection of the TTS1 gene target. (B) Dynamic range analysis of the real-time PCR assay's detection of the TTS1 gene target. The regression line represents data in the linear range. Error bars equal two standard deviations of the aggregate mean. The slope, correlation coefficient (R^2), and PCR efficiency (E) are displayed.

Mean assay results were used to establish an LLOD of 8.4×10^3 CFU/ml (Fig. 2A) and the LDR of 5.4×10^4 to 5.4×10^8 CFU/ml (Fig. 2B). The regression coefficient ($R^2 = 0.99$) and the amplification efficiency value ($E = 1.17$) demonstrated an exponential amplification of DNA with this probe and primer pair.

DISCUSSION

Melioidosis is a significant public health burden in regions of Southeast Asia and northern Australia. Septicemic melioidosis, if left untreated, is a rapidly progressing infection that has a mortality rate as high as 90% (45). In regions not endemic for melioidosis *Burkholderia pseudomallei* is also of concern because of its potential release during a biothreat event. Therefore, we developed a real-time PCR for rapid identification of *B. pseudomallei* isolates that potentially offers a faster and more reliable diagnosis of disease in regions endemic for melioidosis. Rapid identification of *B. pseudomallei* is especially important in these regions because of the high prevalence of *B. pseudomallei* strains that are resistant to the antibiotics typically used to empirically treat sepsis (20, 45). Other methods of rapid identification such as latex agglutination are not commercially available. Here we have described the development and evaluation of a rapid, sensitive real-time assay for the specific identification of *B. pseudomallei*. The real-time assay targeting *orf2* of the *B. pseudomallei* type III secretion system accurately distinguished a large sample of diverse *B. pseudomallei* isolates from non-*B. pseudomallei* isolates (100% specificity) and was not inhibited by human blood products or DNA.

A number of rapid presumptive biochemical and PCR assays have been developed and evaluated for the identification of *B. pseudomallei* (14, 16, 21, 27, 32, 51). The molecular methods for identification of *B. pseudomallei* infection include a number of conventional PCRs with various specificities that also may suffer from a lack of sensitivity (16, 22, 32, 51). More recently two real-time PCR assays have been developed and well evaluated on bacterial isolates (36, 38, 39). Both real-time assays had greater sensitivity than the traditional PCR assays and appear specific for the identification of isolates of *B. pseudomallei* or the *B. pseudomallei*/*B. mallei* complex. This increased sensitivity suggests that they may be suitable for clinical samples, but both assays may require DNA or cell preassay enrichment to detect bacteremic infections. There have been no reported evaluations of either assay for diagnosis of melioidosis in a clinical setting.

Estimates of the concentration of *B. pseudomallei* in blood for septicemic melioidosis patients range from 1 to 1,000 CFU per ml (33, 37, 41). The onset of melioidosis symptoms for a given blood cell count varies depending on a combination of isolate virulence and host factors (9, 10, 35). Based on experiments with *B. pseudomallei*-spiked blood the TTS1 real-time assay has a 95% probability of detecting a concentration of 8.4×10^3 CFU/ml with a single PCR (Fig. 2A). Following analysis of DNA extracted from multiple dilutions of *B. pseudomallei*-spiked human blood samples, the lowest concentration that yielded at least one positive target amplification was 500 CFU/ml, equaling a 12.5% probability of detection (Fig. 2A). By increasing the assay volume of extracted clinical DNA in this system fivefold and testing multiple replicates, the probability of detecting a positive clinical sample using the TTS1 real-time assay is likely to improve. Assuming a Poisson distribution of sample DNA, a clinical specimen with ~500 cells/ml has an extrapolated probability of detection of 100% in at least two of three replicate samples with this fivefold increase in sample volume.

These results suggest that early bacteremia may be beyond the limit of detection of the current assay conditions. Data presented here were based on DNA extracted from spiked blood samples; no attempt to concentrate the cells or DNA was made prior to extraction. Early detection may be accomplished by assaying a concentrated sample either through preprocessing of a blood specimen or collecting a clinical specimen with a higher cell concentration such as sputum or wound exudate from a focal infection site (B. Currie, personal communication). An adjusted assay reduces the time for presumptive diagnosis from days to 3 h, allowing for early definitive antibiotic intervention and minimizing laboratorian contact time.

The power of a well-designed and evaluated real-time PCR assay is demonstrated by its ability to detect target DNA in the

presence of a vast excess of other nontarget DNA (24). During outbreaks of melioidosis associated with severe weather events such as cyclones, after natural disasters such as tsunamis, or following a bioterror release, large numbers of patients could potentially overwhelm the capacity of a health care facility (1, 10). A reliable single target assay could rapidly screen large numbers of patients using multiple specimens from usually sterile (blood or urine), and nonsterile (wound, throat, or sputum) clinical sites to identify those needing immediate antibiotic therapy for melioidosis (34).

Testing environmental samples containing diverse microbial ecology may be enhanced by the use of a selective identification technique such as the TTS1 real-time PCR, but this requires further evaluation. Also, large-scale environmental surveys to establish ecological distribution and concentration using this assay would be a benefit in endemic areas for melioidosis.

The real-time PCR assay targeting *orf2* of the *B. pseudomallei* type III secretion system is a powerful tool for the rapid identification of *B. pseudomallei* and can potentially complement current confirmatory diagnostic procedures for melioidosis. Further studies to evaluate the TTS1 real-time assay on a larger panel of environmental samples, including an evaluation of more *B. thailandensis* strains, are needed. This less-virulent close relative of *B. pseudomallei* is a common soil saprophyte in the hyperendemic melioidosis region of northeast Thailand (4, 11). The evaluation of rapid diagnostic assays using clinical samples in a region of melioidosis endemicity is necessary for an accurate assessment of the clinical efficacy of proposed new diagnostic tools and is under way as part of an ongoing prospective melioidosis cohort study in northern Australia.

ACKNOWLEDGMENTS

REFERENCES

1. **Allworth, A. M.** 2005. Tsunami lung: a necrotising pneumonia in survivors of the Asian tsunami. Med. J. Aust. **182:**364.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Ashdown, L. R.** 1992. Melioidosis and safety in the clinical laboratory. J. Hosp. Infect. **21:**301–306.
4. **Brett, P., D. DeShazer, and D. Woods.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48:**317–320.
5. **Centers for Disease Control and Prevention.** 2004. Laboratory exposure to *Burkholderia pseudomallei*—Los Angeles, California, 2003. Morb. Mortal. Wkly. Rep. **53:**988–990.
6. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.
7. **Currie, B. J.** 2003. Melioidosis: an important cause of pneumonia in residents of and travellers returned from endemic regions. Eur. Respir. J. **22:**542–550.
8. **Currie, B. J., D. A. Fisher, D. M. Howard, J. N. Burrow, D. Lo, S. Selva-Nayagam, N. M. Anstey, S. E. Huffam, P. L. Snelling, P. J. Marks, D. P. Stephens, G. D. Lum, S. P. Jacups, and V. L. Krause.** 2000. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. Clin. Infect. Dis. **31:**981–986.
9. **Currie, B. J., D. A. Fisher, D. M. Howard, J. N. Burrow, S. Selvanayagam, P. L. Snelling, N. M. Anstey, and M. J. Mayo.** 2000. The epidemiology of melioidosis in Australia and Papua New Guinea. Acta Trop. **74:**121–127.
10. **Currie, B. J., and S. P. Jacups.** 2003. Intensity of rainfall and severity of melioidosis, Australia. Emerg. Infect. Dis. **9:**1538–1542.
11. **Dance, D. A.** 2000. Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia* spp. and human-animal hosts. Acta Trop. **74:**159–168.
12. **Dance, D. A. B.** 2000. Melioidosis as an emerging global problem. Acta Trop. **74:**115–119.
13. **Gee, J. E., C. T. Sacchi, M. B. Glass, B. K. De, R. S. Weyant, P. N. Levett, A. M. Whitney, A. R. Hoffmaster, and T. Popovic.** 2003. Use of 16S rRNA gene sequencing for rapid identification and differentiation of *Burkholderia pseudomallei* and *B. mallei*. J. Clin. Microbiol. **41:**4647–4654.
14. **Glass, M. B., and T. Popovic.** 2005. Preliminary evaluation of the API 20NE and RapID NF plus systems for rapid identification of *Burkholderia pseudomallei* and *B. mallei*. J. Clin. Microbiol. **43:**479–483.
15. **Green, T. A., C. M. Black, and R. E. Johnson.** 1998. Evaluation of bias in diagnostic-test sensitivity and specificity estimates computed by discrepant analysis. J. Clin. Microbiol. **36:**375–381.
16. **Haase, A., M. Brennan, S. Barrett, Y. Wood, S. Huffam, D. O'Brien, and B. Currie.** 1998. Evaluation of PCR for diagnosis of melioidosis. J. Clin. Microbiol. **36:**1039–1041.
17. **Hoffmaster, A. R., R. F. Meyer, M. D. Bowen, C. K. Marston, R. S. Weyant, K. Thurman, S. L. Messenger, E. E. Minor, J. M. Winchell, M. V. Rassmussen, B. R. Newton, J. T. Parker, W. E. Morrill, N. McKinney, G. A. Barnett, J. J. Sejvar, J. A. Jernigan, B. A. Perkins, and T. Popovic.** 2002. Evaluation and validation of a real-time polymerase chain reaction assay for rapid identification of *Bacillus anthracis*. Emerg. Infect. Dis. **8:**1178–1182.
18. **Holden, M. T. G., R. W. Titball, S. J. Peacock, A. M. Cerdeno-Tarraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. F. Oyston, and J. Parkhill.** 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. USA **101:**14240–14245.
19. **Inglis, T. J., D. Chiang, G. S. Lee, and L. Chor-Kiang.** 1998. Potential misidentification of *Burkholderia pseudomallei* by API 20NE. Pathology **30:**62–64.
20. **Jenney, A. W., G. Lum, D. A. Fisher, and B. J. Currie.** 2001. Antibiotic susceptibility of *Burkholderia pseudomallei* from tropical northern Australia and implications for therapy of melioidosis. Int. J. Antimicrob. Agents **17:**109–113.
21. **Koh, T. H., L. S. Yong Ng, J. L. Foon Ho, L. H. Sng, G. C. Wang, and R. V. Tzer Pin Lin.** 2003. Automated identification systems and *Burkholderia pseudomallei*. J. Clin. Microbiol. **41:**1809. (Letter.)
22. **Lew, A. E., and P. M. Desmarchelier.** 1994. Detection of *Pseudomonas pseudomallei* by PCR and hybridization. J. Clin. Microbiol. **32:**1326–1332.
23. **Lowe, P., C. Engler, and R. Norton.** 2002. Comparison of automated and nonautomated systems for identification of *Burkholderia pseudomallei*. J. Clin. Microbiol. **40:**4625–4627.
24. **Mackay, I. M.** 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. **10:**190–212.
25. **Monastyrskaya, G., A. Fushan, I. Abaev, O. Filyukova, M. Kostina, E. Pecherskih, and E. Sverdlov.** 2004. Genome-wide comparison reveals great inter- and intraspecies variability in *B. pseudomallei* and *B. mallei* pathogens. Res. Microbiol. **155:**781–793.
26. **Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer, and D. E. Woods.** 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect. Immun. **72:**4172–4187.
27. **O'Brien, M., K. Freeman, G. Lum, A. C. Cheng, S. P. Jacups, and B. J. Currie.** 2004. Further evaluation of a rapid diagnostic test for melioidosis in an area of endemicity. J. Clin. Microbiol. **42:**2239–2240.
28. **Rainbow, L., C. A. Hart, and C. Winstanley.** 2002. Distribution of type III secretion gene clusters in *Burkholderia pseudomallei*, *B. thailandensis* and *B. mallei*. J. Med. Microbiol. **51:**374–384.
29. **Reischl, U., H. J. Linde, N. Lehn, O. Landt, K. Barratt, and N. Wellinghausen.** 2002. Direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila* in clinical specimens by dual-color real-time PCR and melting curve analysis. J. Clin. Microbiol. **40:**3814–3817.
30. **Reischl, U., M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch, and N. A. Strockbine.** 2002. Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **40:**2555–2565.
31. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.

32. **Sermswan, R., S. Wongratanacheewin, N. Anuntagool, and S. Sirisinha.** 2000. Comparison of the polymerase chain reaction and serologic tests for diagnosis of septicemic melioidosis. Am. J. Trop. Med. Hyg. **63:**146–149.

33. **Simpson, A., P. Howe, V. Wuthiekanun, and N. White.** 1999. A comparison of lysis centrifugation, pour plate, and conventional blood culture methods in the diagnosis of septicaemic melioidosis. J. Clin. Pathol. **52:**616–619.

34. **Sirisinha, S., N. Anuntagool, T. Dharakul, P. Ekpo, S. Wongratanacheewin, P. Naigowit, B. Petchclai, V. Thamlikitkul, and Y. Suputtamongkol.** 2000. Recent developments in laboratory diagnosis of melioidosis. Acta Trop. **74:**235–245.

35. **Smith-Vaughan, H. C., D. Gal, P. M. Lawrie, C. Winstanley, K. S. Sriprakash, and B. J. Currie.** 2003. Ubiquity of putative type III secretion genes among clinical and environmental *Burkholderia pseudomallei* isolates in northern Australia. J. Clin. Microbiol. **41:**883–885.

36. **Thibault, F. M., E. Valade, and D. R. Vidal.** 2004. Identification and discrimination of *Burkholderia pseudomallei*, *B. mallei*, and *B. thailandensis* by real-time PCR targeting type III secretion system genes. J. Clin. Microbiol. **42:**5871–5874.

37. **Tiangpitayakorn, C., S. Songsivilai, N. Piyasangthong, and T. Dharakul.** 1997. Speed of detection of *Burkholderia pseudomallei* in blood cultures and its correlation with the clinical outcome. Am. J. Trop. Med. Hyg. **57:**96–99.

38. **Tomaso, H., T. L. Pitt, O. Landt, S. A. Dahouk, H. C. Scholz, E. C. Reisinger, L. D. Sprague, I. Rathmann, and H. U. Neubauer.** 2005. Rapid presumptive identification of *Burkholderia pseudomallei* with real-time PCR assays using fluorescent hybridization probes. Mol. Cell. Probes **19:**9–20.

39. **Tomaso, H., H. C. Scholz, S. Al Dahouk, T. L. Pitt, T. M. Treu, and H. Neubauer.** 2004. Development of 5′ nuclease real-time PCR assays for the rapid identification of the *Burkholderia mallei*/*Burkholderia pseudomallei* complex. Diagn. Mol. Pathol. **13:**247–253.

40. **Vaerman, J. L., P. Saussoy, and I. Ingargiola.** 2004. Evaluation of real-time PCR data. J. Biol. Regul. Homeost. Agents **18:**212–214.

41. **Walsh, A. L., M. D. Smith, V. Wuthiekanun, Y. Suputtamongkol, W. Cha-**

owagul, D. A. Dance, B. Angus, and N. J. White. 1995. Prognostic significance of quantitative bacteremia in septicemic melioidosis. Clin. Infect. Dis. **21:**1498–1500.

42. **Walsh, A. L., M. D. Smith, V. Wuthiekanun, Y. Suputtamongkol, V. Desakorn, W. Chaowagul, and N. J. White.** 1994. Immunofluorescence microscopy for the rapid diagnosis of melioidosis. J. Clin. Pathol. **47:**377–379.

43. **Walsh, A. L., and V. Wuthiekanun.** 1996. The laboratory diagnosis of melioidosis. Br. J. Biomed. Sci. **53:**249–253.

44. **Weyant, R. S., C. W. Moss, R. Weaver, D. Hollis, J. Jordan, E. Cook, and M. Daneshvar.** 1996. Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria, 2nd ed. Williams and Wilkins, Baltimore.

45. **White, N. J.** 2003. Melioidosis. Lancet **361:**1715–1722.

46. **Winstanley, C., B. A. Hales, and C. A. Hart.** 1999. Evidence for the presence in *Burkholderia pseudomallei* of a type III secretion system-associated gene cluster. J. Med. Microbiol. **48:**649–656.

47. **Winstanley, C., and C. A. Hart.** 2000. Presence of type III secretion genes in *Burkholderia pseudomallei* correlates with Ara⁻ phenotypes. J. Clin. Microbiol. **38:**883–885.

48. **Wuthiekanun, V., N. Anuntagool, N. J. White, and S. Sirisinha.** 2002. Short report: a rapid method for the differentiation of *Burkholderia pseudomallei* and *Burkholderia thailandensis*. Am. J. Trop. Med. Hyg. **66:**759–761.

49. **Wuthiekanun, V., V. Desakorn, G. Wongsuvan, P. Amornchai, A. C. Cheng, B. Maharjan, D. Limmathurotsakul, W. Chierakul, N. J. White, N. P. Day, and S. J. Peacock.** 2005. Rapid immunofluorescence microscopy for diagnosis of melioidosis. Clin. Diagn. Lab Immunol. **12:**555–556.

50. **Wuthiekanun, V., Y. Suputtamongkol, A. J. H. Simpson, P. Kanaphun, and N. J. White.** 2001. Value of throat swab in diagnosis of melioidosis. J. Clin. Microbiol. **39:**3801–3802.

51. **Zysk, G., W. D. Splettstosser, and H. Neubauer.** 2000. A review on melioidosis with special respect on molecular and immunological diagnostic techniques. Clin. Lab. **46:**119–130.