

Highly Sensitive Direct Detection and Quantification of *Burkholderia pseudomallei* Bacteria in Environmental Soil Samples by Using Real-Time PCR^{∇†}

Trinh Thanh Trung,¹ Adrian Hetzer,¹ André Göhler,¹ Eysin Topfstedt,¹ Vanaporn Wuthiekanun,² Direk Limmathurotsakul,^{2,3} Sharon J. Peacock,^{2,4,5} and Ivo Steinmetz^{1*}

Friedrich Loeffler Institute of Medical Microbiology, Ernst Moritz Arndt University of Greifswald, Greifswald, Germany¹; Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand²; Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand³; Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand⁴; and Department of Medicine, Cambridge University, Addenbrooke's Hospital, Cambridge, United Kingdom⁵

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The soil bacterium and potential bioterror agent *Burkholderia pseudomallei* causes the infectious disease melioidosis, which is naturally acquired through environmental contact with the bacterium. Environmental detection of *B. pseudomallei* represents the basis for the development of a geographical risk map for humans and livestock. The aim of the present study was to develop a highly sensitive, culture-independent, DNA-based method that allows direct quantification of *B. pseudomallei* from soil. We established a protocol for *B. pseudomallei* soil DNA isolation, purification, and quantification by quantitative PCR (qPCR) targeting a type three secretion system 1 single-copy gene. This assay was validated using 40 soil samples from Northeast Thailand that underwent parallel bacteriological culture. All 26 samples that were *B. pseudomallei* positive by direct culture were *B. pseudomallei* qPCR positive, with a median of 1.84×10^4 genome equivalents (range, 3.65×10^2 to 7.85×10^5) per gram of soil, assuming complete recovery of DNA. This was 10.6-fold (geometric mean; range, 1.1- to 151.3-fold) higher than the bacterial count defined by direct culture. Moreover, the qPCR detected *B. pseudomallei* in seven samples (median, 36.9 genome equivalents per g of soil; range, 9.4 to 47.3) which were negative by direct culture. These seven positive results were reproduced using a nested PCR targeting a second, independent *B. pseudomallei*-specific sequence. Two samples were direct culture and qPCR negative but nested PCR positive. Five samples were negative by both PCR methods and culture. In conclusion, our PCR-based system provides a highly specific and sensitive tool for the quantitative environmental surveillance of *B. pseudomallei*.

The Gram-negative betaproteobacterium *Burkholderia pseudomallei*, a natural inhabitant of soil and surface water, causes the infectious disease melioidosis, with high mortality rates for infected humans in tropical and subtropical regions where the disease is endemic (7, 10, 58). Clinical cases of melioidosis have been reported regularly in Southeast Asian countries, such as Thailand (28, 53), Vietnam (42), and Malaysia (43, 62), in northern and Western Australia (12, 19), and sporadically in India (1, 49), South and Central America (20, 45), and West and East Africa (21). The disease can be acquired by inoculation of *B. pseudomallei* through skin lesions, by aerosols from contaminated soil and surface water, or by ingestion (11, 15). Melioidosis has attracted interest outside its known distribution areas by cases in travelers (9) and soldiers (32, 40) returning from regions of *B. pseudomallei* endemicity and by its classification as a category B bioterrorism agent (47). In many known areas

and countries of endemicity where melioidosis is likely to occur, diagnostic resources in the clinical laboratory are limited, and therefore, the true burden of the disease and the worldwide distribution of *B. pseudomallei* remain unclear.

Apart from the detection of *B. pseudomallei* in clinical cases, knowledge of the distribution and lifestyle of *B. pseudomallei* in its natural soil environment is important for understanding the epidemiology of melioidosis. In this context, quantitative detection of *B. pseudomallei* is crucial for investigating its associations with specific habitats, as well as the influence of factors such as climate change. Several investigations have used culture-based quantification (39, 50) to enumerate *B. pseudomallei* bacteria within its natural environment. Quantitative cultivation of *B. pseudomallei* from soil samples depends on efficient detachment of microorganisms from the soil matrix, which relies on the selected dispersion method (56). However, detection of *B. pseudomallei* by culture can be hindered by the presence and overgrowth of other environmental bacterial species capable of growing on the currently used selective media (6), especially when only low *B. pseudomallei* cell numbers are present. Additionally, the proportion of *B. pseudomallei* environmental cells which might be viable but are in a nonculturable state under standard laboratory conditions is unknown. It seems likely that this phenomenon, described for other envi-

* Corresponding author. Mailing address: Friedrich Loeffler Institute of Medical Microbiology, Ernst Moritz Arndt University of Greifswald, Martin-Luther-Strasse 6, 17475 Greifswald, Germany. Phone: 49 3834 865587. Fax: 49 3834 865561. E-mail: steinmetz.ivo@uni-greifswald.de.

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TABLE 1. Bacterial isolates used in this study

Type of isolate	Species	No. of isolates	Origin	Reference(s) or source
Reference ^a	<i>B. pseudomallei</i> K96243	1	Female diabetic patient, Thailand	17
	<i>B. pseudomallei</i> E8	1	Rice field soil, North Thailand	61
	<i>Burkholderia thailandensis</i> DSM13276 ^T	1	Rice field soil, Central Thailand	5
	<i>Burkholderia vietnamiensis</i> LMG 18835	1	Cystic fibrosis isolate, USA	26
	<i>Burkholderia cepacia</i> H111	1	Cystic fibrosis isolate, Germany	16, 46
Clinical ^a	<i>B. pseudomallei</i>	25	Northern Vietnam	42
Environmental ^b	<i>B. pseudomallei</i>	2	Northern Vietnam	This study
	<i>B. thailandensis</i>	44	Northern Vietnam	This study
	<i>B. cepacia</i>	1	Northern Vietnam	This study
	<i>B. seminalis</i>	1	Northern Vietnam	This study
	<i>B. pyrocinia</i>	1	Northern Vietnam	This study
	<i>B. diffusa</i>	4	Northern Vietnam	This study
	<i>B. latens</i>	5	Northern Vietnam	This study
	<i>B. vietnamiensis</i>	12	Northern Vietnam	This study
	<i>R. solanacearum</i>	2	Northern Vietnam	This study
Total		102		

^a Collection of strains deposited at the Friedrich Loeffler Institute of Medical Microbiology, Greifswald University, Greifswald, Germany.

^b Strains isolated from soil collected in northern Vietnam in 2006.

ronmental species (2, 36), also contributes to an underestimation of the *B. pseudomallei* bacterial load or to false-negative results from environmental habitats.

Molecular methods based on direct bacterial nucleic acid extraction from environmental samples and subsequent amplification have the potential to overcome many restrictions of traditional microbiological approaches but are associated with other pitfalls (57). Efficient DNA isolation from soil is biased by incomplete cell lysis and nucleic acid adsorption to soil particles (13, 25, 31). Furthermore, soil-derived PCR inhibitors coextracted with nucleic acids affect downstream amplification reactions (38, 57). Although a multitude of DNA extraction and purification methods exist, including several commercially available kits, there is no universal standard protocol and methods have to be adapted to the specific experimental needs.

The present study was undertaken to develop a quantitative PCR method for the direct quantification of *B. pseudomallei* cells from soil. We first established a protocol for efficient DNA extraction and the removal of coextracted amplification inhibitors. *B. pseudomallei* cells were then detected as genome equivalents (GE) in a quantitative PCR (qPCR) using primers and a probe, specific to a 115-bp fragment of the type three secretion system 1 (TTSS1) of *B. pseudomallei*, which have recently been developed to detect this pathogen in both clinical samples and supernatants from environmental enrichment cultures (23, 24, 33). A nested PCR targeting a second *B. pseudomallei*-specific sequence (52) was applied to qualitatively confirm the qPCR results. This experimental approach was validated on 40 environmental soil samples collected in Northeast Thailand and proved to be a highly sensitive tool for rapid environmental surveillance of *B. pseudomallei*, compared to culture methods.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used are listed in Table 1. Bacteria stored in frozen vials were streaked onto Columbia agar plates supplemented with 5% sheep blood (Becton Dickinson, Heidelberg, Germany).

The plates were then incubated overnight at 37°C in air. Fresh cultures grown on agar were harvested for bacterial DNA extraction.

Environmental strains were isolated from soil samples collected in northern Vietnam in 2006 using the traditional method based on soil dispersion in water according to Smith et al. (50). The colonies on Ashdown's agar plates were screened for *B. pseudomallei* morphotypes (6). Suspected *B. pseudomallei* isolates were identified by oxidase test and a specific latex agglutination test (51). *B. pseudomallei* isolates and representatives of non-*B. pseudomallei* colonies were further identified by sequencing the 16S rRNA gene and the *recA* gene (22, 41). Sequence similarity searches were performed using the BLAST algorithm implemented in the NCBI database (<http://www.ncbi.nlm.nih.gov>).

Soil samples. The PCR methods were validated on sandy loam soil samples collected in January (samples S 01 to S 95) or March (samples RFT 01 to RFT 30) 2010 from randomly selected rice fields with known high positivity rates for *B. pseudomallei* in Amphoe Lao Sua Kok, Ubon Ratchathani province, Northeast Thailand, as described previously (56). Briefly, a standard soil sampling technique was used (29), and 200 g of soil was collected from a depth of 30 cm. Soil samples sealed in plastic bags and plastic containers were shipped by air freight at ambient temperatures to the laboratory in Greifswald, Germany, and stored at room temperature until analyzed, because low temperatures are able to reduce the viable cell count of *B. pseudomallei* (55). Samples S 01 to S 95 and RFT 01 to RFT 30 were processed for DNA extraction (see below) within 10 days after soil sampling in Northeast Thailand. There was no significant difference in the qPCR-based *B. pseudomallei* detection (see below) between subsamples extracted on different days (data not shown). The original 200-g soil samples were mechanically homogenized prior to the experiments, and three 1-g, 0.5-g, or 0.1-g subsamples of each original 200-g sample were processed for DNA extraction and purification using different methods as described below. For culture experiments, three 25-g subsamples were processed as previously described (56). Additionally, 10 soil samples were taken from a 30-cm depth from agricultural land around Greifswald, Germany, as negative controls from an area of nonendemism to confirm the specificity of the PCR methods and to test for cross-contamination.

Culture-based detection of *B. pseudomallei* cells in environmental soil samples.

The single PCR data of the 40 soil samples from this study were compared to the single culture results of the same samples, previously reported as summarized data by Trung and colleagues (56). Briefly, *B. pseudomallei* cells were detached from the soil matrices of 25-g subsamples by shaking in 50 ml of a solution containing 2.5% (wt/vol) polyethylene glycol 6000 (SERVA Electrophoresis, Heidelberg, Germany) and 0.1% (wt/vol) sodium deoxycholate (Merck KGaA, Darmstadt, Germany) for 2 h. The soil particles were then sedimented by centrifugation at 1,400 × g for 10 min. One hundred microliters of the supernatant was used to enumerate *B. pseudomallei* cells on Ashdown's agar (containing 10 g of Trypticase soy broth, 40 ml of glycerol, 5 ml of 0.1% crystal violet, 5 ml of 1% neutral red, 5 mg of gentamicin, and 15 g of agar per liter). For a qualitative

TABLE 2. Primers and probes used for the noncompetitive amplification control, the TTSS1 gene qPCR assay, and the nested PCR

Purpose	Primer or probe	Oligonucleotide sequence (5'-3') ^a	Amplified DNA fragment	Source or reference
Detection of PCR inhibitors	IAC2 forward IAC2 reverse CMV3 probe	GAATTCGCCCTTATTAGCCGAC GGAATTCGCCCTTTAATGCGC HEX-TGATCGGCGTTATCGCGTTCTTGATC-BHQ2	Insertion sequence of pCR2.1-IAC	This study
qPCR target	BpTT4176 forward BpTT4290 reverse BpTT4208 probe	CGTCTCTATACTGTGCGAGCAATCG CGTGCACACCGGTCAAGTATC FAM-CCGGAATCTGGATCACCACCACCTTTCC-BHQ1	TTSS1 gene/5' region (-82 to +34) of BPSS1407 <i>setD</i> ^{b,c}	37
Nested-PCR target	174 outer forward 725 outer reverse 8653 inner forward 8653 inner reverse 8653 inner probe	ACCTTTCGTCGGCATGGTAG GCCCGCTTCTGGTCTTTATTC ATCGAATCAGGGCGTTCAAG CATTCGGTGACGCACACGACC FAM-CGCCGCAAGACGCCATCGTTCAT-BHQ1	BPSS1187 ^d coding region ^b BPSS1187 ^d coding region ^b	This study 52

^a HEX, hexachloro-6-carboxyfluorescein; BHQ2, black hole quencher 2; FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1.

^b Coding sequence denoted for the complete genome sequence of *B. pseudomallei* K96243 (17).

^c The NCBI accession number of BPSS1407 *setD* is NC_006351 (region 1920833 to 1921882).

^d The NCBI accession number of BPSS1187 is NC_06351 (region 1593544 to 1594350).

culture, 1 ml of the same supernatant was mixed with 9 ml of Galimand broth (threonine-basal salt plus colistin [TBSS-C50]) (61), supplemented with 1 g/liter of polymyxin B. Enrichment cultures were incubated at 40°C for 4 days before being plated on Ashdown's agar plates. All plates were incubated at 40°C for 4 days, and the numbers of *B. pseudomallei* CFU were determined.

DNA extraction from soil and subsequent purification. Total genomic DNA was extracted from mechanically homogenized soil samples by two commercially available kits according to the manufacturers' instructions, except where noted, and by the extraction method developed in this study.

The SoilMaster DNA extraction kit (Epicentre Biotechnologies, Madison, WI) was used according to the manufacturer's instructions. Briefly, 0.1 g of soil was added to 250 μ l of soil DNA extraction buffer. Then bacterial cell lysis and DNA purification were performed using a hot detergent lysis process and a column chromatography step, respectively. The final precipitated DNA preparation was resuspended in 50 μ l of Tris-EDTA (TE) buffer and stored at -20°C until used.

The FastDNA spin kit for soil (MP Biomedicals, Illkirch, France) was applied according to the manufacturer's instructions. Briefly, 0.5 g of soil was added to the lysing matrix A tube, which contained 987 μ l of sodium phosphate buffer, 122 μ l of MT buffer (from the Fast DNA spin kit), and a mixture of ceramic and silica particles. Then the bacterial cell disruption and DNA purification steps were performed using a vortex and a silica-based procedure, respectively. The final DNA preparation was eluted in 50 μ l of DNase-pyrogen-free water and stored at -20°C until used.

The DNA extraction part of the protocol developed in this study is based on the protocol of Gabor et al. (14), with slight modifications. First, 1 g of soil was mixed with 750 μ l of lysis buffer (100 mM Tris HCl, 100 mM Na₂EDTA, 1.5 M NaCl, 1% cetyltrimethyl ammonium bromide [CTAB], pH 8.0) by vortexing the mixture at maximal speed until the soil was completely homogenized. After the addition of 40 μ l of lysozyme (50 mg ml⁻¹) and 10 μ l of proteinase K (10 mg ml⁻¹), the tubes were incubated at 37°C for 30 min. Then 200 μ l of SDS (20% wt/vol) was added prior to incubation at 65°C for 2 h, with a vigorous vortex for several seconds after 1 h of incubation. The first supernatant (supernatant A) was collected by centrifugation at 6,000 \times g for 10 min, and the soil pellet was reextracted by adding 1 ml of lysis buffer, in contrast to the original protocol (14). Vortexing for a few seconds was followed by incubation at 65°C for 30 min. Centrifugation was performed at 6,000 \times g for 10 min, and the second supernatant (supernatant B) was collected and combined with supernatant A. Instead of chloroform (14), an equal volume of chloroform-isoamyl alcohol (49:1) was added to the mixture before the DNA was precipitated from the upper water phase by an addition of 0.6 volume of isopropanol and incubated at -20°C overnight (or at least 1 h). The DNA precipitate was collected by centrifugation at 16,000 \times g for 7 min, washed with prechilled 70% ethanol, and resuspended in 50 μ l of TE buffer, followed by incubation at room temperature for 1 h.

To purify DNA from potential coextracted PCR inhibitors, a modified form of Moreira's protocol (35) was used. Crude soil DNA was gently mixed with an equal amount of melted 1.6% low-melting-point (LMP) agarose (AppliChem, Darmstadt, Germany) in a sterile 2-ml tube. After the tube was allowed to stand at 4°C for 15 min, the solidified DNA-agarose matrix was subjected to three washing steps, with each step performed by adding 1.5 ml of TE buffer to the tube

and placing the tube horizontally at 4°C for 24 h. After the last washing step, the purified DNA remained embedded in the LMP agarose matrix and was stored at -20°C. Before incorporation into the PCR, the DNA-agarose block was incubated at 70°C for 2 min and the melted solution was used as a template. Initial experiments revealed that the melted agarose solution did not affect PCR performance (data not shown).

Detection of PCR inhibitors in purified soil DNA. A quantitative PCR assay was developed in order to detect the presence of PCR inhibitors coextracted with nucleic acids in the soil DNA extracts and to validate the quality of the different soil DNA extraction methods. The plasmid pCR2.1-IAC was constructed by inserting an artificial synthesized DNA fragment (MWG-Biotech, Ebersberg, Germany) of 135 bp (AGCCGACACGCGTCTCTATACTGTGCGAGCAATCG GCGGATATCCCCTCACGCTGTTGTGATCGGCGTATCGCGTTCTCGGTTCTTGA TCGCACTTTACGAAGCTGTTACGATACTGACCGGTGTGACGCGG GCGCGCA), containing a unique nucleotide sequence of cytomegalovirus (indicated in bold), into a pCR2.1 plasmid (Invitrogen, Darmstadt, Germany). The quantitative PCR assay of the recombinant plasmid pCR2.1-IAC had the same thermal conditions as the TTSS1 target amplification. One hundred copies of pCR2.1-IAC were incorporated into a 25- μ l reaction mixture as described for the following TTSS1 gene qPCR assay (see below), except that the primers and the probe were replaced with a 400 nM concentration each of primers IAC2 forward and IAC2 reverse and a 260 nM concentration of CMV3 probe (MWG-Biotech, Ebersberg, Germany), labeled with hexachloro-6-carboxyfluorescein (HEX) at its 5' end and black hole quencher 2 (BHQ2) at its 3' position (Table 2). The thermal cycling profile was as described for the TTSS1 qPCR assay (see below), except that the number of thermal cycles was set to 60. The degree of PCR inhibition caused by soil DNA extracts was determined by cycle threshold differences (ΔC_T), which were calculated by subtracting the C_T values of the PCR with the recombinant plasmid and soil DNA extracts from the C_T values of the PCR mixture containing the recombinant plasmid as a single template (control).

Quantitative TTSS1 PCR for the detection of *B. pseudomallei*. The TTSS1 gene qPCR mixture, at a final volume of 25 μ l, consisted of 1 \times TaqMan universal PCR master mix (Applied Biosystem, Branchburg, NJ), which contained the following: AmpliTaq Gold DNA polymerase and AmpErase uracil-N-glycosylase (UNG); a 400 nM concentration each of primer BpTT4176 forward and BpTT4290 reverse; a 260 nM concentration of BpTT4208 probe (MWG-Biotech, Ebersberg, Germany), labeled with 6-carboxyfluorescein (FAM) at its 5' end and black hole quencher 1 (BHQ1) at its 3' position (Table 2); 10 μ g of nonacetylated bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO); and 4 μ l of purified soil DNA embedded in an agarose matrix as a template. Amplification and detection were performed on the Mx3000P qPCR system (Stratagene, Cedar Creek, TX) using the manufacturer's standard settings. Thermal conditions were 50°C for 2 min to activate UNG, which prevents carryover contamination, followed by an initial denaturation step at 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 s and amplification at 60°C for 1 min. The cycle threshold (C_T) values were automatically calculated by applying adaptive baseline algorithms (MXPro-Mx3000P v. 320, build 340). The primers used were empirically tested and produced no artifacts (data not shown).

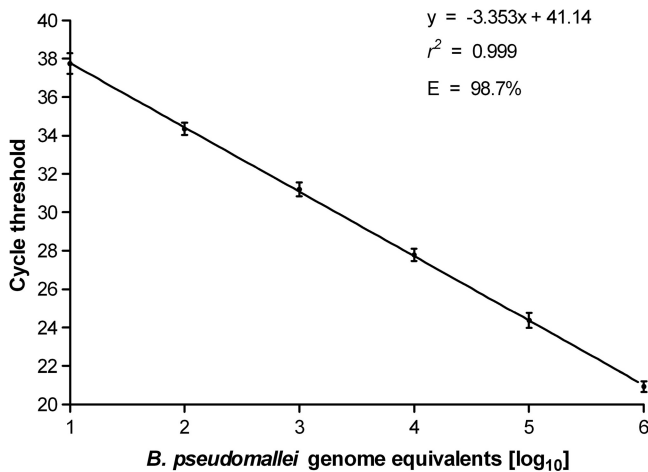


FIG. 1. Straight calibration line based on genomic *B. pseudomallei* DNA for TTSS1 gene qPCR. The equation and the square regression coefficient (r^2) of the standard curve and the efficiency (E) of the PCR are given. Values are the means of nine independent determinations \pm standard errors of the mean (SEM) (error bars).

BPSS1187 gene nested PCR for the detection of *B. pseudomallei*. To further improve the sensitivity of *B. pseudomallei* detection and to reduce the effect of any remaining PCR inhibitors, a nested-PCR approach was applied. The outer primers, 174 forward (174F) and 725 reverse (725R) (Table 2), were used to amplify a 552-bp fragment of BPSS1187, which encodes a hypothetical *B. pseudomallei*-specific protein (according to the genome sequence of *B. pseudomallei* K96243) (52). The first PCR was performed in a 25- μ l reaction mixture which consisted of 1 \times PCR buffer containing 1.5 mM MgCl₂, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystem, Foster City, CA), a 125 μ M concentration each of deoxynucleotides dATP, dCTP, dGTP, and dTTP (Roche, Mannheim, Germany), a 400 nM concentration each of primers 174F and 725R (Table 2), 10 μ g of BSA, and 4 μ l of purified soil DNA immobilized in solid agarose as a template. Thermal cycling was carried out in an Uno II thermocycler (Biometra, Goettingen, Germany), with an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min. One microliter of the resulting PCR product was then applied as the template in a second PCR using the 8563 PCR assay (Table 2) as described by Supaprom and colleagues (52). A melting-curve analysis was performed using the SYBR green I master kit (Roche Applied Science, Mannheim, Germany) to check for primer-dimer artifact formation.

Determining sensitivities, specificities, and efficiencies of the PCRs by using pure bacterial cultures. To determine the detection limits and efficiencies of the TTSS1 qPCR genomic DNA from freshly grown cultures of *B. pseudomallei*, *B. pseudomallei* strain K96243 was isolated using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Quantification was performed using SYBR green I master mix (Stratagene, Cedar Creek, TX) and lambda DNA as the standard (molecular weight marker XV; Roche, Mannheim, Germany), by following the manufacturer's protocol, on the Mx3000P qPCR system (Stratagene, Cedar Creek, TX). The amount of DNA was then converted to genome equivalent (GE) copies based on the *B. pseudomallei* K96243 genome size of 7.25 Mb (17):

$$\text{No. of GE copies} = \frac{(6.02 \times 10^{23} \text{ copies/mol}) \times \text{DNA amount (g)}}{7.25 \times 10^6 \text{ bp} \times (660 \text{ g/mol bp})} \quad (1)$$

Serially diluted DNA was used as a template to generate nine individual standard curves for each real-time PCR assay (Fig. 1). To validate the specificities of the qPCR assay, the nested-PCR genomic DNA was isolated from bacterial cultures of 29 *B. pseudomallei* strains (Table 1) and 73 strains closely related phylogenetically to *B. pseudomallei* (Table 1) as described above. Furthermore, all primers were bioinformatically compared with published *Burkholderiales* sequences. By use of the Primer-BLAST tool (48), implemented on the NCBI website with default parameters and with exclusion of sequences with four or more mismatches, a specificity of primers for only *B. pseudomallei* sequences was revealed. Both probes shared full-length identity only with sequences of *B. pseudomallei*. The comparison of the TTSS1 primers and probe with the TTSS of *Ralstonia solanacearum* CFBP2957 plasmid RCFBPv3_mp (44) (GenBank ac-

cession no. FP885907.1) showed sequence similarities only with gaps and mismatches of more than five nucleotides within the alignments (8) (data not shown).

PCR-based detection and quantification of *B. pseudomallei* from soil. For the detection of *B. pseudomallei* in soil samples by using either TTSS1 qPCR or BPSS1187 nested PCR, each of the three 1-g subsamples was analyzed in duplicate. TTSS1 qPCR bacterial counts of each replicate, expressed as genome equivalents (GE) per PCR mixture, were determined with the standard curve shown in Fig. 1. The bacterial count per g of soil was calculated with the following equation:

$$\begin{aligned} \text{No. of GE copies/g of soil} &= [(50 \mu\text{l eluate} + 50 \mu\text{l LMP agarose})/\text{g of soil}] \\ &\times [(\text{no. of GE copies/PCR mix})/(4 \mu\text{l eluate-LMP mix/PCR mix})] \end{aligned} \quad (2)$$

Statistical analysis. The statistical significance of differences was determined by two-tailed Student's *t* tests and Wilcoxon signed rank tests with GraphPad Prism software version 4.0 for Windows (GraphPad Software, San Diego, CA). The quartile coefficient of dispersion (interquartile range/median) was used to specify the intrasample variation of qPCR results obtained in the three PCR runs, each performed in duplicate.

RESULTS

Detection of coisolated PCR inhibitors in soil DNA by using different extraction methods. We first evaluated the quality of genomic DNA with respect to coisolated PCR inhibitors after using different extraction methods in 10 soil samples from Northeast Thailand. These samples were culture positive for *B. pseudomallei*. The genomic DNA was isolated with either the SoilMaster DNA extraction kit, the FastDNA spin kit for soil, or our method based on enzymatic and chemical methods plus subsequent purification in an agarose matrix. A noncompetitive internal amplification control was applied to determine the rate of inhibition. The differences in cycle threshold (ΔC_T) values between PCRs with and without soil DNA extracts, supplemented with 100 recombinant plasmid copies, were used to determine the grade of inhibition. All three tested DNA isolation methods failed to completely remove PCR inhibitors coisolated with genomic DNA from soil. Table 3 (columns without BSA) shows that accurate detection of the copy numbers of the incorporated recombinant plasmid failed for 6 samples (60%) isolated by the SoilMaster DNA extraction kit, with either no amplification at all or very high ΔC_T values, and for all 10 samples (100%) obtained from both the FastDNA spin kit for soil and our developed protocol. The majority of soil DNA extracts showing no qPCR amplifications were brownish in color, indicating the presence of coextracted soil compounds interfering with the qPCR assay. Additional washing steps using either 5.5 M guanidine thiocyanate or humic acid wash solution, optional in the FastDNA spin kit, did not improve PCR performance for DNA samples isolated by this kit (data not shown).

Significant reduction of PCR inhibitory effects of soil compounds. We overcame PCR inhibition by the incorporation of nonacetylated BSA into the qPCR assay as follows. All 26 samples that were initially qPCR negative displayed amplification in the presence of BSA (Table 3, columns with BSA). The ΔC_T values for each of the three tested extraction protocols, with 10 soil samples each, were significantly reduced using BSA as a PCR adjuvant, with overall ΔC_T values of 0.48 ± 0.09 , 0.47 ± 0.22 , and 0.85 ± 0.17 for the SoilMaster DNA

TABLE 3. Presence of PCR inhibitory effects after using different soil DNA extraction protocols

Sample	ΔC_T^a					
	SoilMaster kit		FastDNA kit		Protocol of this study	
	Without BSA	With BSA	Without BSA	With BSA	Without BSA	With BSA
S 01	Inhibited	0.88 ± 0.55	Inhibited	1.65 ± 0.08	Inhibited	1.47 ± 0.23
S 03	Inhibited	0.32 ± 0.06	Inhibited	0.74 ± 0.20	Inhibited	1.21 ± 0.19
S 04	Inhibited	0.93 ± 0.12	Inhibited	1.74 ± 0.27	Inhibited	1.49 ± 0.15
S 05	0.86 ± 0.31	0.32 ± 0.13	>6.55 ^b	0.01 ± 0.18	Inhibited	0.74 ± 0.26
S 06	13.47 ± 1.38	0.33 ± 0.06	Inhibited	0.08 ± 0.04	Inhibited	1.13 ± 0.12
S 07	1.59 ± 0.14	0.27 ± 0.06	Inhibited	0.04 ± 0.05	Inhibited	0.68 ± 0.32
S 10	1.31 ± 0.23	0.07 ± 0.10	Inhibited	0.06 ± 0.10	Inhibited	-0.19 ± 0.26
S 12	2.45 ± 0.45	0.59 ± 0.08	Inhibited	-0.06 ± 0.21	Inhibited	0.47 ± 0.20
S 92	Inhibited	0.79 ± 0.15	Inhibited	0.57 ± 0.26	Inhibited	1.22 ± 0.15
S 95	0.64 ± 0.11	0.36 ± 0.03	>4.83 ^b	-0.11 ± 0.30	Inhibited	0.30 ± 0.11
Overall ΔC_T		0.48 ± 0.09		0.47 ± 0.22		0.85 ± 0.17

^a The degree of PCR inhibition is reflected by the cycle threshold difference (ΔC_T), which was calculated by subtracting C_T values of the PCR of soil DNA extracts with the incorporation of a noncompetitive internal amplification control (IAC) from C_T values of the PCR mixture containing the IAC as a single template (control). PCRs with ΔC_T values greater than 2 were considered to be strongly inhibited (boldface). Data are the means and standard errors of the means (SEM) of results from three independent experiments. "Inhibited" indicates that no PCR amplification signal could be detected in three subsamples after 60 cycles.

^b PCR amplification signal could be detected in only one or two subsamples after 60 cycles.

extraction kit, the FastDNA spin kit for soil, and our developed protocol, respectively (Table 3, columns with BSA).

Sensitivity and specificity of TTSS1 gene qPCR and BPSS1187 gene nested PCR. We then optimized a TTSS1 gene qPCR assay which should subsequently be used for the direct quantitative detection of *B. pseudomallei* in soil. Figure 1 shows the straight calibration line relating the C_T values to the numbers of *B. pseudomallei* genome equivalents by using a 10-fold dilution series of genomic *B. pseudomallei* K96243 DNA. There was a strong linear inverse relationship ($r^2 = 99.9\%$) between the C_T values and the \log_{10} genome equivalents of *B. pseudomallei* over 6 orders of magnitude. Higher variations of the C_T values were observed if fewer than 10 genome equivalents per PCR mixture were used. Therefore, 10 *B. pseudomallei* genome equivalents per PCR mixture was used as the lower limit for calculation. The limit of detection (LOD) was 3 *B. pseudomallei* genome equivalents per single qPCR mixture, corresponding to 75 *B. pseudomallei* cells per g of soil when assuming a DNA extraction efficiency of 100%.

The efficiency of qPCR amplification was 98.7%. To ensure accurate quantification of the qPCR, melting-curve analyses were conducted, which confirmed the absence of any primer-dimers (data not shown).

To qualitatively confirm any TTSS1-based qPCR signal by a second PCR target in case of negative results in culturing, we also established a BPSS1187 gene-based nested PCR. The LOD of this assay was 1 GE/PCR mixture with a used volume of 5 μ l. This corresponds to a theoretical value of 20 GE per g of soil for the qualitative detection of *B. pseudomallei*. The specificity of the TTSS1 primers and probe and the specificity of the inner BPSS1187 primers and probe for *B. pseudomallei* strains have already been rigorously tested in previous studies (24, 37, 52). In accordance with these results, our extended testing of the primers and probes of both PCR methods against 73 isolates of species that are closely related phylogenetically, including species such as *Burkholderia seminalis*, *Burkholderia latens*, *Burkholderia diffusa*, and *Burkholderia pyrrocinia* (listed in Table 1; data not shown), revealed no PCR amplification,

whereas all 29 different *B. pseudomallei* isolates proved to be positive in our assays.

Comparison of results of *B. pseudomallei* DNA detection from soil by different DNA extraction methods. To finally select the DNA extraction protocol to be used for the qPCR-based quantification of *B. pseudomallei* in soil, we compared the results of the TTSS1 qPCR with added BSA for five DNA soil samples (S 03 to S 07) processed by the three different DNA extraction methods. Despite the lower average ΔC_T values obtained for the two commercially available kits, our newly developed method led to a higher detection rate of *B. pseudomallei* genome equivalents, indicating a better template quality or quantity (Fig. 2). The detection factors for the SoilMaster DNA extraction kit and FastDNA spin kit for soil were

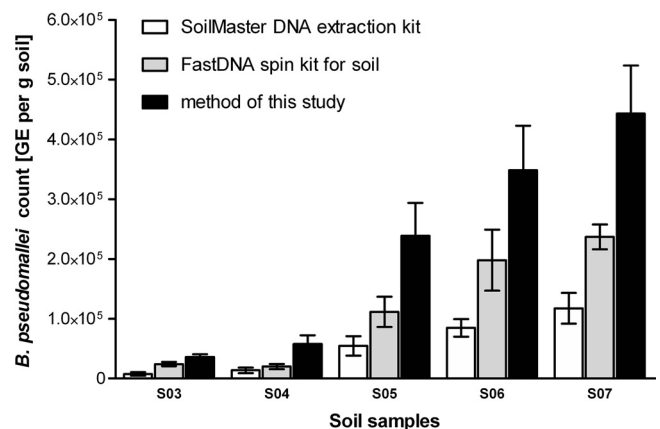


FIG. 2. Comparison of *B. pseudomallei* DNA recoveries from soil for the SoilMaster DNA extraction kit, the FastDNA spin kit for soil, and the extraction method of this study. DNA was isolated from five soil samples, and *B. pseudomallei* genome equivalents were quantified by the TTSS1 qPCR assay with added BSA. The data are the mean values ± SEM (error bars) from three independent experiments, each performed in duplicate.

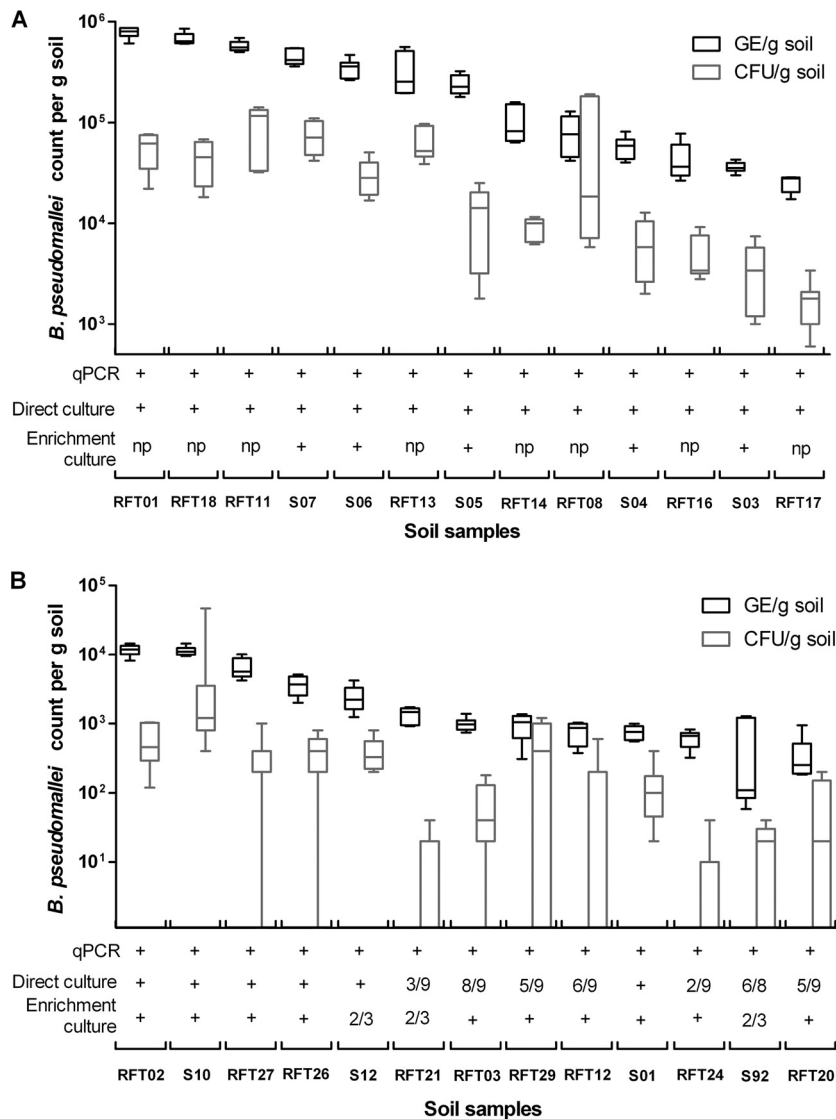


FIG. 3. Quantitative DNA-based detection of *B. pseudomallei* in 26 direct quantitative culture-positive soil samples collected in Northeast Thailand. (A) Samples with values above 25,000 GE per g of soil; (B) samples with values from 300 to 25,000 GE per g of soil. The data of qualitative and quantitative cultures of single samples are from experiments reported in summary in a parallel study in which two culture protocols were compared (56). The given GE and CFU values per g of soil represent the median, the 25th and 75th quartiles, and the range of all replicates of the subsamples. Fractions show the number of positive replicates out of all replicates. “+” indicates detection of *B. pseudomallei* in all replicates. np, not performed.

0.24 ± 0.01 times and 0.52 ± 0.05 times that of our method, respectively.

PCR-based quantification of *B. pseudomallei* in environmental soil samples from Northeast Thailand. Our PCR systems were validated using 40 environmental soil samples collected in Northeast Thailand. The same samples were used in a parallel study in which we validated our newly developed protocol for the culture-based detection of *B. pseudomallei* based on soil dispersion in a polyethylene glycol and sodium deoxycholate solution (56). In addition, we tested 10 soil samples from Germany as negative controls from an area of nonendemicity. A sample was classified as PCR positive when *B. pseudomallei* DNA could be detected in at least one replicate of the three 1-g subsamples. The results in Fig. 3 show that all 26 samples

from Northeast Thailand which were *B. pseudomallei* positive by direct quantitative culture were *B. pseudomallei* qPCR positive (Fig. 3A, 13 samples with >25,000 GE/g of soil; Fig. 3B, 13 samples with 300 to 25,000 GE/g of soil), with a median of 1.84×10^4 genome equivalents per gram of soil (range, 3.65×10^2 to 7.85×10^5). In those 26 samples, raw data of colony counts were poorly linearly related to qPCR results ($r^2 = 0.629$), whereas log transformation of culture and qPCR data resulted in a high correlation ($r^2 = 0.96$). All samples exhibited a ΔC_T value below 2 (data not shown), indicating no significant inhibition of qPCR amplification. Moreover, determination of total DNA by fluorescent dye staining or of total bacterial DNA by bacterial 16S rRNA gene copies from representative soil samples did not correlate with the quantity of *B. pseu-*

TABLE 4. PCR-based detection of *B. pseudomallei* in 14 quantitative direct culture-negative soil samples collected in Northeast Thailand^a

Group	Sample	Bacterial count from qPCR ^c (mean no. of GE/g of soil ± SEM)	No. of positive replicates/total no. of replicates ^b			
			qPCR	BPSS1187 gene nested PCR	Enrichment culture ^d	Direct culture
I	RFT 04	129 (174)	3/9	+	+	–
	RFT 28	47 ^e (62)	3/9	4/6	1/3	–
	S 95	37 ^e (28)	2/8	+	1/3	–
	RFT 05	11 ^e (27)	1/9	4/6	1/3	–
	RFT 23	–	–	4/6	2/6	–
II	RFT 22	509 (610)	2/9	+	–	–
	RFT 15	22 ^e (54)	1/9	+	–	–
	RFT 10	9 ^e (23)	1/9	2/6	–	–
	RFT 19	–	–	4/6	–	–
	RFT 06	–	–	–	–	–
	RFT 07	–	–	–	–	–
	RFT 09	–	–	–	–	–
	RFT 25	–	–	–	–	–
	RFT 30	–	–	–	–	–

^a The data of enrichment cultures of single samples are from experiments reported in summary in a parallel study in which two culture protocols were compared (66). The bacterial counts, determined by qPCR as the numbers of GE copies per g of soil, represent the means of all replicates of the subsamples. Samples were grouped as follows: group I, positive enrichment culture; group II, negative enrichment culture.

^b +, detection of *B. pseudomallei* in all replicates; –, no detection of *B. pseudomallei* in any replicates.

^c The data are the means and SEM of the results of three independent PCR experiments performed in duplicate (first subsample of S 95) or triplicate. Calculations were performed using equation 2. –, no bacterial count was obtained.

^d Culture results on Ashdown's agar after a 4-day enrichment step in Galimand broth at 40°C.

^e GE value was extrapolated using the equation shown in Fig. 1.

domallei GE (data not shown), indicating that the degree of *B. pseudomallei* DNA detection was not generally linked to the amount of bacterial DNA present or to the efficiency of recovering bacterial DNA from single samples.

Importantly, the qPCR assay yielded, on average, 10.6-fold (geometric mean; range, 1.1-fold to 151.3-fold) higher cell numbers than the respective CFU counts per gram obtained with our newly developed culture protocol based on soil dispersion in a polyethylene glycol and sodium deoxycholate solution (56). The qPCR-based cell count was 42.8-fold higher (geometric mean; range, 14-fold to 276-fold) than the cell count of the less sensitive standard soil culture protocol based on soil dispersion in water (50, 56). These differences were significant for both culture protocols when they were compared to qPCR, with *P* values below 0.0001 (Wilcoxon signed rank test).

Only three 1-g subsamples were used for the qPCR out of the original 200-g sample, compared to the three 25-g subsamples used for culture experiments (56). Nonetheless, the intrasample dispersion (quartile coefficient of dispersion) of *B. pseudomallei* detection was significantly lower (*P* = 0.0007; Wilcoxon signed rank test) for the qPCR results (median, 0.33; range, 0.1 to 7.47) than for the direct culture results (median, 0.98; range, 0.38 to 9.36), as determined by Trung et al. (56).

The PCR results of the samples which were negative by direct culture but positive by enrichment culture or which were negative by both culture methods are shown in Table 4. Out of five samples positive only by enrichment culture and negative by direct culture (Table 4, group I), four samples were qPCR positive, with a median of 4.2×10^1 genome equivalents, and another sample was positive only by the nested PCR.

Out of nine samples negative by both culture methods (Table 4, group II), three samples were positive by qPCR, one

sample was positive by nested PCR only, and five samples were negative by both PCR methods.

In other words, the increased sensitivity of the qPCR compared to that of the quantitative direct culture was demonstrated by detection of *B. pseudomallei* in seven samples which were negative by direct culture, as shown in Table 4 (33 [94%] qPCR positive versus 26 [74%] direct quantitative culture positive, *P* = 0.045, Fisher's exact test). Those seven samples were all positive by nested PCR and, in the case of four samples, positive in enrichment cultures, supporting the qPCR results. Templates extracted and purified from the 10 German soil samples were negative, as expected, in the TTSS1 qPCR assay (data not shown), as *B. pseudomallei* is not endemic in that country, providing further confirmation of the specificity of the PCR assays.

DISCUSSION

Although described almost a century ago (60), the worldwide environmental distribution of *B. pseudomallei* is still unknown and our understanding of the environmental factors determining the presence of *B. pseudomallei* is rudimentary. As a basis for a better understanding of *B. pseudomallei* ecology in its natural habitat, quantitative culture-dependent and quantitative culture-independent molecular methods are needed to detect the organism. The two methodological approaches are likely to complement rather than replace each other. To the best of our knowledge, the direct quantitative detection of *B. pseudomallei* from an environmental habitat using molecular methods has not been reported. We therefore aimed to establish a quantitative DNA-based method to detect *B. pseudomallei* in soil samples from an area of endemicity.

The soil samples used for validation of our PCR methods originated from randomly selected rice fields in Amphoe Lao Sua

Kok, Ubon Ratchathani province, Northeast Thailand, and consisted of sandy loam taken at a depth of 30 cm. This habitat is normally anoxic except for the rhizosphere of the rice plants (27), where members of the order *Burkholderiales* can also be found (30). Generally, *Proteobacteria* represent a minor group of the whole microbial community in bulk rice field paddies (27).

Direct molecular bacterial detection from soil is methodologically challenging because PCR inhibitors are often coextracted with the DNA. Depending on the soil type, different concentrations of inhibitory components can be found. These include humic acid, fulvic acid, polysaccharides, and metal ions (59), all of which negatively affect the DNA polymerase activity and/or the availability of DNA templates (38). Various strategies have been proposed for excluding or reducing inhibitory effects in soil samples prior to PCR, such as the use of cesium chloride density gradient ultracentrifugation (4), dialysis (3), cetyltrimethylammonium bromide (CTAB) for complexing inhibitors, polyvinylpyrrolidone (PVPP) (63), chromatography, electrophoresis, or multivalent cations (59) or the procedures of separating inhibitors from nucleic acids applying gel filtration (34), washing DNA immobilized in agarose (35), or using PCR additives, such as nonacetylated bovine serum albumin (BSA) and phage T4 gene 32 protein (59).

PCR inhibition was also observed in this study for the two commercial soil DNA isolation kits and the protocol developed in this study. Although both commercial kits tested coupled DNA extraction with purification by either chromatography (SoilMaster DNA extraction kit) or silica (FastDNA spin kit for soil), inhibition of amplification was not significantly prevented until BSA was added to the PCR reagent mix. This was also true for our protocol, which combined a conventional, slightly modified DNA extraction protocol with the washing of extracted DNA embedded in an agarose matrix. However, using this protocol together with BSA as a PCR additive resulted in the highest sensitivity for *B. pseudomallei* detection in soil with a TTSS1-based qPCR.

A limitation of our study is the restriction to soil samples of sandy loam only, which is the most prevalent soil type in Northeast Thailand. Therefore, further field studies have to demonstrate the general usefulness of our protocol for different soil types. For the calculation of the sensitivity of our PCR methods, we assumed a theoretical DNA extraction efficiency of 100%, being well aware that the soil type might influence the efficiency of bacterial DNA extraction. We addressed this issue in preliminary experiments in which we quantified total bacterial DNA by 16S rRNA gene copies in soil samples collected around Greifswald in Northeast Germany and in representative samples from Northeast Thailand. Our results revealed a comparable, even slightly higher bacterial load in the soil around Greifswald, including samples with clay loam- and silty loam-like textures (data not shown), indicating a potential usefulness of our protocol for other soil types.

Previous studies targeted the TTSS1 gene and BPSS1187 for the qualitative detection of *B. pseudomallei* in enrichment cultures of soil samples (24) and clinical samples (52), respectively. The results of our study confirm the specificity of these targets, since the TTSS1 gene and the BPSS1187 coding sequence were present in all *B. pseudomallei* bacteria tested. Furthermore, these sequences could not be detected within genomic DNA of closely related species. Importantly, the

TTSS1 qPCR protocol developed in this study led to an improved PCR efficiency and a wider linear range of *B. pseudomallei* detection compared to those of a PCR protocol applied for the detection of *B. pseudomallei* in environmental enrichment cultures, with the same TTSS1 gene sequence used as the target (24).

Combining the TTSS1 qPCR assay with the BPSS1187 gene nested approach, we could classify 35 out of 40 soil samples as *B. pseudomallei* positive. Out of these *B. pseudomallei*-positive samples, 26 samples were positive using our recently improved quantitative culture-based method (56) and therefore were directly compared to our qPCR protocol developed in this study. The significantly higher numbers of *B. pseudomallei* bacteria detected in those 26 soil samples by TTSS1 qPCR (Fig. 3) might be explained by the coextraction of extracellular DNA and/or DNA originating from viable but nonculturable cells of the bacterium (18). Although the subsamples used for PCR consisted of much less soil material than the subsamples used for the culture method, the TTSS1 qPCR resulted in a significantly lower intrasample dispersion. It seems likely that an uneven distribution in the culturable proportion of *B. pseudomallei* populations, which might be due to differences in the hydration status of the soil as well as in the soil type itself (54), is responsible for this phenomenon.

Thirty (96.8%) out of 31 samples which were positive by either direct culture or enrichment culture resulted in a positive signal in the TTSS1 qPCR assay. The only exception was sample RFT 23, in which case the culture after the enrichment step was positive for *B. pseudomallei* but the TTSS1 gene amplification was negative. However, this sample tested positive in the nested BPSS1187 PCR. Out of the 35 *B. pseudomallei*-positive samples, the TTSS1 qPCR detected more positive samples than did quantitative direct culture (33 [94%] versus 26 [74%], $P = 0.045$, Fisher's exact test). However, with our sample size, the qualitative sensitivities were not significantly different when we compared the 35 samples which were positive by either qPCR or nested BPSS1187 PCR to the 31 samples which were positive by either direct or enrichment culture ($P = 0.39$, Fisher's exact test).

In conclusion, our presented qPCR method is able to detect significantly larger numbers of *B. pseudomallei* bacteria within soil samples, with a lower dispersion of subsample results, than direct culturing methods. The nested-PCR approach detects *B. pseudomallei* in samples at a detection limit that is below that of the qPCR and thereby is able to further improve the culture-independent sensitivity of *B. pseudomallei* detection in soil. Taken together, our experimental system will likely help unravel the ecology of *B. pseudomallei* in its natural habitat.

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