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Clinically Practical Seminested PCR for *Burkholderia pseudomallei* Quantitated by Enzyme Immunoassay with and without Solution Hybridization

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Diagnosis of melioidosis, an infectious disease caused by *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*), is made initially by antibody testing, which is not always sensitive or specific. We have developed two seminested PCR protocols combined with enzyme immunoassay (EIA) to detect the conserved ribosomal regulatory region of *B. pseudomallei*. Both PCRs used one biotinylated primer for capturing PCR products on EIA plates. One system, termed solution hybridization EIA (SHEIA), hybridized PCR products with a digoxigenin-labeled probe in solution. Another system, termed primer-labeled EIA (PLEIA), used a digoxigenin-labeled nested primer to generate products that were directly detected without hybridization. To prevent amplicon contamination, pre-PCR uracil DNA glycosylase treatment or post-PCR UV irradiation was incorporated into each system. By a rapid method of blood sample preparation for PCR, these systems had sensitivities of 75 bacteria per ml for SHEIA and 300 bacteria per ml for PLEIA. No nonspecific amplification of other bacterial DNAs was detected. This seminested PCR coupled with SHEIA or PLEIA fulfills all the requirements for a diagnostic test to be used in developing countries where *B. pseudomallei* is endemic.

Burkholderia pseudomallei, formerly known as *Pseudomonas pseudomallei* (23), is the causative agent of melioidosis, a lethal infectious disease endemic in Southeast Asia and northern Australia. Known as one of the great imitators, melioidosis can clinically resemble other bacterial infections. Misdiagnosis and mistreatment are common (8). Diagnosis of melioidosis is made initially by clinical awareness and is supported by antibody testing. Serology is not always sensitive in acute cases, as most patients are immunocompromised. In addition, high background titers from asymptomatic infections in areas where *B. pseudomallei* is endemic complicate the use of the antibody assay in diagnosing and monitoring the efficacy of treatment (5). Isolation of the organism makes a definitive diagnosis, but up to 2 weeks may be required to culture the organism (15). Therefore, a PCR-based nucleic acid detection system could prove to be a useful diagnostic tool.

Although PCR has broad scientific applications, its use as a clinical diagnostic tool requires reliability, rapidity, economy, and results that can be quantitated and standardized. Furthermore, development of a clinically useful PCR diagnostic test must take into account (i) appropriate processing of clinical specimens, (ii) an efficient PCR protocol coupled with a method for prevention of PCR product contamination, and (iii) a clinically practical method for detecting PCR products (7). One of the risks of using PCR in the clinical laboratory is the possibility of contamination of subsequent samples with the amplified products of previous PCRs. To reduce this risk, techniques which enable previously generated PCR products to be readily destroyed before a new sample is processed have been devised (1, 10, 11, 14). One such technique employs the substitution of dUTP for dTTP, which renders the amplified products susceptible to destruction by uracil DNA glycosylase (UDG) (4, 10). Alternatively, UV irradiation can also cross-link PCR products, preventing them from serving as templates for subsequent amplifications (11).

In this report, we describe two sensitive protocols for the detection of *B. pseudomallei* in blood samples. For this purpose, blood samples were treated by a rapid protocol. Seminested PCR was used to generate products that were quantitatively detected by enzyme immunoassay (EIA). Two PCR-EIA systems, with and without solution hybridization, were explored in these studies. For the prevention of amplicon contamination, different decontamination methods, UDG or UV treatment, that were appropriate for each system were incorporated into these protocols.

MATERIALS AND METHODS

Bacteria. *B. pseudomallei* strains isolated from 35 patients at Khonkaen University Hospital (6 from blood cultures, 2 from pleural fluid samples, 2 from urine samples, 15 from exudates obtained from abscesses, and 11 from sputum samples) were subcultured on blood agar for 2 days, harvested, washed, and resuspended in phosphate-buffered saline (PBS) with 0.1% glutaraldehyde to kill bacteria. Killed bacteria were lyophilized for transport to the United States, where they were reconstituted to the same volume with water. The other bacteria used (*Burkholderia cepacia* [formerly Pseudomonas cepacia] [23], *Pseudomonas aeruginosa*, *Pseudomonas vesicularis*, *Xanthomonas maltophilia*, *Shewanella putrefaciens*, *Comamonas testosteroni*, *Escherichia coli*, *Enterobacter cloacae*, *Acinetobacter calcoaceticus* var. *anitratus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* complex) were kindly provided by the Clinical Microbiology Laboratory, Johns Hopkins Hospital. Bacterial DNAs were extracted and measured for concentrations according to a previously described method (17).

Processing of whole blood for PCR. *B. pseudomallei* was suspended in blood and prepared for PCR by a modification of a previously described method (9) for processing up to 1 ml of whole blood. The reconstituted *B. pseudomallei* suspension was first diluted with PBS at an optical density at 550 nm (OD_{550}) equivalent to the no. 4 McFarland standard (2.4×10^9 cells per ml) and further diluted to make 1.2×10^3, 6×10^2, 3×10^2, 1.5×10^2, and 75 cells per ml with EDTA-anticoagulated human blood. Then 1 ml of each blood sample was centrifuged in

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a 1.5-ml tube at 12,000 × *g* for 5 min. Plasma was discarded. Packed blood cells were lysed with 0.5 ml of 0.1% (wt/vol) saponin by gentle rotation at 37°C for 15 min and then centrifuged at 12,000 × *g* for 10 min; once again, the supernatant was discarded. The pellet was lysed again with saponin as described above, and the resulting bacterial pellet was mixed with 100 μl of chelex suspension (40% [wt/wt] chelex-100 [Bio-Rad, Hercules, Calif.] in 0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Nonidet P-40, 1% [vol/vol] Tween 20) (2), vortexed, and incubated at 95°C for 15 min. Then each sample was stored at 4°C, frozen, or assayed by PCR. Before PCR, the sample was vortexed and centrifuged at 12,000 × *g* for 5 s, and 10 μl of supernatant was put into the PCR mixture.

PCR primers. Oligonucleotide primers specific for *B. pseudomallei* were selected after the DNA sequences of the internal transcribed spacers between 16S and 23S rRNA genes (18) from *B. pseudomallei*, *B. cepacia*, *Burkholderia* (*Pseudomonas*) *mallei*, *Burkholderia* (*Pseudomonas*) *gladioli*, and *P. aeruginosa* had been aligned by using MegAlign software (DNAstar, Madison, Wis.). bp1 (5′-CGATGATCGTTGGCGCTT; positions 397 through 414 [numbered according to reference 18]) and bp4 (5′-CGTTGTGCCGTATTCCAAT; positions 698 through 680) are outer primers, while bp2 (5′-ACTTACGGGCATCTCA; positions 420 through 435) and bp3 (5′-ATTAGAGTCGAACAAT; positions 681 through 666) are inner primers nested inside the bp1-bp4 region. These primer sequences were chosen because they are absent or different from the sequences of other bacteria, except that of *B. mallei*; *B. pseudomallei* and *B. mallei* have similar DNA sequences spanning the 16S to 23S rRNA genes, including this internal transcribed spacer (9, 18, 23). The bp1 primer used in all experiments was 5′ labeled with biotin. The bp3 primer used in seminested PCR for detection by primer-labeled EIA (PLEIA) was 5′ labeled with digoxigenin (DIG). All primers were custom synthesized and labeled (Bio-Synthesis, Lewisville, Tex.).

Seminested PCR with pre-PCR UDG decontamination for detection by SHEIA. All PCR mixtures were prepared on an ice bath to prevent primer-dimer formation. The reaction mixture consisted of 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 3.5 mM $MgCl_2$; 0.2 mM (each) dATP, dGTP, dCTP, and dUTP; 2.5 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.); and 0.05 U of UDG (Gibco) in a total volume of 100 μl in a 0.5-ml thin-wall polypropylene tube (Wheaton, Millville, N.J.). The primers for seminested amplification were added as follows: 0.4 μM biotinylated bp1, 0.04 μM bp4, and 0.6 μM bp3. The use of unequal amounts of primers was adapted from a previously described one-tube nested PCR technique (3). The reaction began at 50°C for 5 min to allow UDG to excise the uracil base from any previously generated contaminating PCR products and continued at 94°C for 2 min to break the abasic DNA and complete the denaturing of the template. The cycling profile was as follows: 90°C for 10 s, 65°C for 10 s, and 72°C for 30 s (with a 2-s increase every cycle) for 25 cycles; 90°C for 10 s, 55°C for 10 s, and 72°C for 80 s (with a 2-s increase every cycle) for 35 cycles; and 72°C for 30 min to deactivate UDG. After cycle completion, the tube was kept at 4°C until detection by solution hybridization EIA (SHEIA) or electrophoresis on a 1.5% agarose gel with ethidium bromide staining.

Seminested PCR with post-PCR UV treatment for detection by primer-labeled EIA (PLEIA). The constituents of these PCR mixtures were the same as those described above, except that for detection without hybridization, a DIG-labeled bp3 primer was used and UDG was omitted. The temperature profile described above was adjusted by the following: (i) the initial 50°C step was deleted, (ii) the 55°C annealing temperature of the last 35 cycles was reduced to 40°C, and (iii) the 72°C soaking step after PCR was reduced from 30 to 10 min. For the prevention of amplicon contamination, post-PCR UV treatment was adapted from a previously described protocol (11). After the reaction was completed, the tube was placed at a distance of 2 cm from the light source in a UV cross-linker (Spectroline, Westbury, N.Y.) for 30 min.

Generation of DIG-labeled probe for use in SHEIA. A two-step nested PCR, with outer primers bp1 and bp4 and nested primers bp2 and bp3, was used to generate a DIG-labeled probe for use in SHEIA. An equal amount of each primer (0.2 μM) was added. DIG-dUTP (Boehringer Mannheim, Indianapolis, Ind.) was substituted for 35% of the dUTP in the nested (bp2 and bp3) reaction mixture. *B. pseudomallei* DNA (100 fg) was used in outer amplification for 35 cycles at the 65°C annealing temperature. One microliter of a 1:10 dilution of the outer PCR products was put into the nested reaction mixture to amplify for 40 cycles at a 55°C annealing temperature. The nested PCR products were used as the DIG probe without further purification.

SHEIA for detection of biotin-seminested PCR products. The principles of SHEIA are illustrated in Fig. 1. Two microliters of biotinylated PCR products was added to 400 μl of hybridization solution containing 50% formamide, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 10 mM EDTA, 2% blocking reagent (BR) (Boehringer Mannheim), 0.05% Tween 20, and 0.2 μl of the DIG probe. The solution was mixed, incubated at 95°C for 10 min to denature both PCR products and probes, and then hybridized at 37°C for 30 min. Two hundred microliters was transferred in duplicate to a streptavidin-coated polystyrene plate (Xenopore, Saddle Brook, N.J.) preblocked with 1% BR in maleate buffer (100 mM maleic acid, 150 mM NaCl [pH 7.5]). After transfer, the plate was incubated at 37°C for 30 min and washed four times with maleate buffer–0.05% Tween 20 and two times without detergent. Two hundred microliters of 0.15-μ/ml anti-DIG peroxidase conjugate (Boehringer Mannheim) in maleate buffer with 1% BR and 0.05% Tween 20 was added to each well. The plate was incubated at 37°C for 30 min and washed as described above, and 200 μl of 0.1-g/l tetramethylbenzidine in peroxidase substrate solution (Pierce, Rockford, Ill.) was added. After 30 min, the reaction was stopped with H_2SO_4 and read at OD_{450}. The OD of the blank was subtracted from that of the sample. Linear regression analysis of the log bacterial concentration and OD was calculated by using computer software (SigmaStat; Jandel Scientific, San Rafael, Calif.).

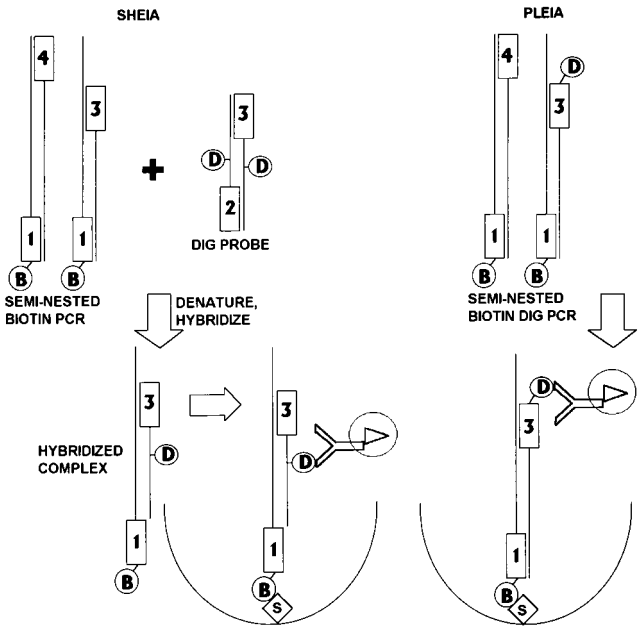

FIG. 1. Illustration of SHEIA (left) and PLEIA (right) for detection of seminested PCR products. B, biotin; D, DIG; S, strepavidin; numbers 1 through 4, primers bp1 through bp4.

PLEIA for detection of biotin-DIG-seminested PCR products. This method was termed PLEIA because both primers of the target product were labeled, one with biotin and the other with DIG. An illustration of PLEIA is also shown in Fig. 1. The streptavidin-coated plate was preblocked with 300 μl of 1% BR in maleate buffer per well at 37°C for 1 h. Then 110 μl of the blocking buffer was taken out. Ten microliters of biotin-DIG-PCR products was added to the plate and mixed by pipetting. The plate was incubated at 37°C for 30 min, washed, and then processed by this detection system, and statistical analysis was performed as described above.

RESULTS

Sensitivity and specificity of primer pair bp1 and bp4. Single-round 35-cycle PCR with primer pair bp1 and bp4 was tested for amplification of 1 ng of DNA from 35 clinical isolates of *B. pseudomallei*. The bp1-bp4 PCR amplified all 35 strains. The bp1-bp4 PCR was tested for specificity also by examining its ability to amplify DNAs from the other bacteria listed in Materials and Methods, including organisms such as *B. cepacia*, which is a closely related species (23). None of these bacteria gave the specific 302-bp PCR product. Only nonspecific high-molecular-weight bands were seen with *B. cepacia*, *P. aeruginosa*, and *S. putrefaciens*. Nevertheless, when DNA was amplified by seminested PCR and detected by SHEIA, all of these bacterial strains gave negative OD results.

Seminested PCR. The intensity of seminested PCR products on an agarose gel correlated with serial dilutions of the DNA template. This PCR detected 100 fg of *B. pseudomallei* DNA (Fig. 2, lanes 3, 9, and 15) on an agarose gel. Separation on a 4% agarose gel revealed that these seminested PCR products consisted of two bands, the 302-bp product of primer pair bp1 and bp4 and the 285-bp product of primer pair bp1 and bp3, which could not be separated on the 1.5% agarose gel usually used (Fig. 3, lanes 1 through 5). This result confirmed the

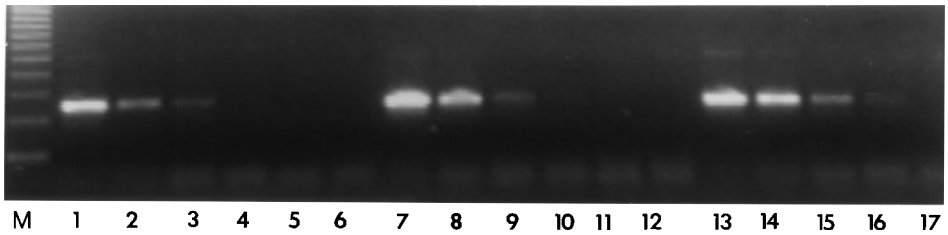

FIG. 2. Seminested PCR amplifications of the following 10-fold serial dilutions of *B. pseudomallei* DNA: 10 pg (lanes 1, 7, and 13), 1 pg (lanes 2, 8, and 14), 100 fg (lanes 3, 9, and 15), 10 fg (lanes 4, 10, and 16), and 1 fg (lanes 5, 11, and 17). Lanes 1 through 6, dTTP was used in PCRs; lanes 7 through 12, dUTP was used; lanes 13 through 17, dUTP and UDG were used. Lane M, 123-bp markers. Lanes 6 and 12, no DNA.

finding of others (22) that outer PCR products predominate in the one-tube (semi- or full-) nested PCR. Both of these bands were correlated with the amount of DNA template. Therefore, it was possible to measure only the inner products for quantitative assay.

In the development of the seminested PCR with DIG-labeled bp3 for direct detection by PLEIA, the 55°C annealing temperature was found to result in weaker intensities for the product bands, especially the 285-bp band (Fig. 3, lanes 6 through 10). Therefore, the 55°C annealing temperature had to be dropped to 40°C to produce an intensity equivalent to that observed with PCR that used unlabeled bp3 (Fig. 3; compare lanes 11 through 15 with lanes 1 through 5) and UDG had to be omitted.

To run 60 cycles of seminested PCR required about 2.5 h, which is a practical run time for a clinical laboratory. The use of a low denaturing temperature (6) and the short duration of each step shortened the total time of this PCR and extended the lives of the reaction components.

Use of UDG-dUTP for carryover decontamination. The seminested PCR with dUTP showed sensitivity comparable to that of PCR with dTTP (Fig. 2; compare lanes 1 through 6 [dTTP] with lanes 7 through 12 [dUTP]). Therefore, dUTP could be substituted for dTTP and used in conjunction with UDG. On the other hand, UDG had no effect on the sensitivity of this seminested PCR (Fig. 2; compare lanes 7 through 12 [no UDG] with lanes 13 through 17 [UDG]).

To test the decontamination ability of UDG, serial dilutions of the seminested PCR products from 1 pg of bacterial DNA template (10^2 initial copies) were reamplified by a new seminested PCR. Without UDG, reamplification of products from 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} μl resulted in smears on the ethidium bromide gel, probably because of an increase in unbalanced forward and reverse primers. However, when UDG was included in reamplification reactions, only a faint smear was observed when 1 μl of products was used as the template and no bands, smears, or SHEIA-positive signals were observed from reamplifying 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} μl of products as the templates. On the basis of normal PCR amplification of 10^2 initial copies for 35 cycles, which produced 10^9 product molecules per μl (1), our seminested PCR amplification of 10^2 initial copies for 60 cycles should contain at least 10^9 product molecules per μl. The finding that less than 10^{-1} μl of products could not be reamplified indicated that 0.05 U of UDG in the reaction mixture destroyed at least 10^8 carryover molecules of our seminested PCR products.

Use of post-PCR UV irradiation for carryover decontamination. Because of the 40°C annealing temperature used in the seminested PCR for PLEIA, UDG could not be used to prevent amplicon contamination. Post-PCR UV irradiation was used instead. This method had the same sterilizing ability as that of UDG. Reamplification of 1 μl of UV-irradiated products amplified from 1 pg of bacterial DNA resulted in a faint smear on the gel. As with UDG, no bands, smears, or PLEIA-positive signals were observed when 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} μl of UV-irradiated products were reamplified. This result also indicated that at least 10^8 carryover molecules of our seminested PCR products were decontaminated by the UV irradiation protocol.

SHEIA and PLEIA. We next tested the abilities of these methods to detect PCR products amplified directly from chelex-treated blood samples. The PCR products amplified from blood samples were quantitatively detected by these two EIA methods. For both systems, ODs increased with increasing concentrations of bacteria in blood samples (Table 1). The ODs by SHEIA were higher than those by PLEIA, and SHEIA was more sensitive than ethidium bromide gel detection. Even the lowest OD by SHEIA (0.2 for 75 bacteria per ml) gave a strong enough reaction to be judged as positive by the naked eye. Negative blood samples had an OD equivalent to that of the blank. The sensitivity of detection was 75 bacteria per ml for SHEIA and 300 bacteria per ml for PLEIA, the same sensitivity gel detection had. Without the additional hybridization step, PLEIA also provided satisfactory specificity, showing that its OD was not affected by primer-dimer artifact products. Linear regression analysis showed that the log of the bacterial concentration correlated significantly with the ODs ($P < 0.005$; adjusted r^2, >0.9) for both EIA systems (Table 1).

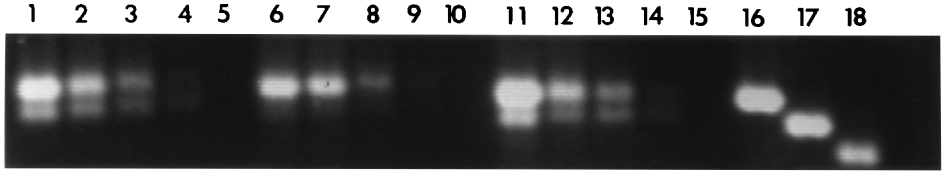

FIG. 3. Seminested PCR products separated on a 4% agarose gel. Lanes 1 through 5, unlabeled bp3 nested primer and 55°C nested annealing temperature were used. Lanes 6 through 10, DIG-labeled bp3 and 55°C nested annealing temperature were used. Lanes 11 through 15, DIG-labeled bp3 and 40°C nested annealing temperature were used. Lane 16, bp1-bp4 PCR product was used as a 302-bp marker. Lane 17, bp1-bp3 PCR product was used as a 285-bp marker. Lane 18, pp2-pp3 PCR product was used as a 262-bp marker.

TABLE 1. OD results from detection of seminested PCR products of blood samples with known concentrations of bacteria and linear regression analysis between ODs and log bacterial concentrations

EIA	OD at concn (bacteria/ml) of:						P^a	Adjusted r^2
	0	75	150	300	600	1,200		
SHEIA	0	0.20	0.86	1.43	2.44	3.34	$<0.001^b$	0.996
PLEIA	0	0	0.08	0.38	0.54	0.70	0.0024^c	0.958

[a] *P* values of the linear regression coefficients.
[b] Linear regression equation: log (bacterial concentration) = 1.83 + (0.382OD).
[c] Linear regression equation: log (bacterial concentration) = 1.94 + (1.53OD).

DISCUSSION

These studies have defined two diagnostic PCR-EIA techniques that can specifically and quantitatively detect *B. pseudomallei*. The first published PCR for *B. pseudomallei* used primers derived from broadly conserved regions in the 23S rRNA gene and subsequent hybridization with a specific probe (9). The PCR technique described here attains specificity through the use of specific primers. The ability of this PCR to amplify all 35 strains of a target species shows how well-conserved the sequences of the primers are in the ribosomal intergenic spacer region. Recent reports indicate that this region is conserved because it is transcribed together with the large- and small-subunit rRNAs in a precursor rRNA and acts as a conserved regulatory signal in ribosomal assembly (20, 21).

The sensitivity levels of 75 bacteria per ml for SHEIA and 300 bacteria per ml for PLEIA make these tests suitable for rapid identification of positive blood cultures (9). However, because *B. pseudomallei* survives and multiplies in human polymorphonuclear and mononuclear phagocytes (13), there may in fact be substantial bacterial numbers in bacteremic blood samples. Therefore, direct analysis of clinical specimens with these techniques may also aid in early diagnosis. This work also demonstrates the quantitative abilities of these tests, showing that the OD results highly correlated with the bacterial loads in samples by linear regression analysis.

Although we have designed a set of four primers for nested PCR, we chose the one-tube seminested PCR for these test systems to avoid amplicon carryover. We also totally substituted dUTP for dTTP from the first use of this set of primers. The inclusion of this compound in the reaction mixture enabled potentially contaminating PCR products to be easily destroyed before the initiation of PCR analysis of subsequent clinical specimens. Contrary to other studies which reported lower sensitivity with dUTP and UDG in PCR (11, 12), we found the same sensitivity as with dTTP. This may be attributable to our use of 20-fold-less UDG than is recommended (10, 11). Reactions were able to destroy at least 10^8 molecules of amplicon. Since most PCR contamination arises from aerosol carryover of less than 10^5 molecules (1, 11), the efficiency of our UDG is sufficient. In a similar manner, Kox et al. (4) used 100-fold-less UDG than is recommended for their PCR. One reason that UDG has not been more widely used is that the standard UDG protocol recommends soaking the reaction mixture at 72°C at the completion of PCR until analysis is performed (14, 16). Alternatively, chloroform extraction of the reaction to deactivate the residual UDG activity has been suggested (19). However, use of an oil-free PCR system, as we did, may result in desiccation of the amplicon if it is soaked for a long time. By using the low UDG concentration, we were able to soak the PCR products at 72°C for only 30 min and then preserve them at 4°C prior to analysis. On the other hand, we also showed the same efficiency of decontamination with UV cross-linking of PCR products which were analyzed without hybridization.

We have demonstrated the detection of *B. pseudomallei* by a sensitive one-tube seminested PCR which does not require the transfer step between outer and inner amplifications, as is necessary for conventional nested PCR. This system utilizes protocols and equipment that are readily available in a clinical diagnostic laboratory. The quantitative ability of this PCR should be useful for establishing a prognosis, determining the type of treatment to be employed, monitoring treatment, and evaluating relapsing infection. Finally, this seminested PCR coupled with direct EIA or SHEIA fulfills all the requirements of a clinically useful diagnostic test and should be suitable for evaluation in routine diagnostic laboratories in developing countries where *B. pseudomallei* is endemic.

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