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PCR combined with lateral flow dipstick assay (PCR-LFD) for a rapid diagnosis of melioidosis

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Abstract

Background: Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*. Septicemic melioidosis patients have a high mortality rate within 48 hours.

Objectives: To develop a polymerase chain reaction (PCR) combined with a lateral flow dipstick (LFD) assay for detection of *B. pseudomallei* in blood samples.

Methods: The PCR with *wcbG* gene primers and a PCR-LFD test were developed. The specificity and sensitivity were determined using the *B. pseudomallei* and other bacterial DNAs. They were evaluated using 43 *B. pseudomallei* positive blood samples and another 43 blood samples positive for other microbial infections.

Results: The detection limit of the PCR-LFD test was 50 fg of bacterial gDNA or 1.0 CFU per 200 µl of blood. All *B. pseudomallei* were positive while *B. thailandensis* and selected gram-negative bacterial strains were negative. The PCR-LFD gave all positives with all 43 *B. pseudomallei* culture positive patient blood samples and all negative with 43 blood samples that were culture positive for *K. pneumoniae*, *E. gallinarum*, *E. faecium*, *E. coli*, *S. aureus*, *A. baumannii*, *A. hydrophila*, *S. haemolyticus*, *S. pneumoniae*, *P. aeruginosa*, *E. cloacae*, *S. hominis*, *E. aerogenes*, *P. mirabilis*, *C. neoformans*, *C. albicans*, *A. caviae*, *E. faecalis* and *K. variicola*.

Conclusions: The developed PCR-LFD assay provided 100% sensitivity and 100% specificity compared to the conventional blood culture. The technique took only 1.5 hours that is easy and quick to perform compared to the 3-7 days of culture method. The new method of PCR with LFD could facilitate the detection to be a semi-point-of-care testing (POCT).

Key words: *Burkholderia pseudomallei*, polymerase chain reaction, lateral flow dipstick assay, melioidosis, blood samples

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Introduction

Burkholderia pseudomallei is a gram-negative bacterium that causes a disease called melioidosis. In Thailand, this disease is the second most common cause of community-acquired bacteremia, and the mortality rate is as high as 43% in the Northeast Thailand. Sepsis cases may die within 48 hours (h) even with appropriate treatment.[1,2] The identification of *B. pseudomallei* in clinical samples from patients suspected with melioidosis using blood culture is used as the gold standard method.[3,4]

Persons with less experience may fail to differentiate *B. pseudomallei* with other closely related *Pseudomonas* spp. or *Burkholderia* spp. Moreover, the culture method requires at least 3-7 days to identify the bacteria which may be too late and co-infection or contamination can be a big obstacle as growth of *B. pseudomallei* is quite slow.[5–7] Therefore, early identification and treatment are necessary for patients especially in septic cases. Serological techniques have been developed for diagnosis of melioidosis such as indirect hemagglutination (IHA), immunofluorescent assay (IFA), and enzyme linked immunosorbent assay (ELISA). These methods with sensitivity and specificity have not yet been developed for early diagnosis.[8–10] The polymerase chain reaction (PCR), a molecular technique, has been developed but it is not in routine practice due to its low sensitivity for direct detection of *B. pseudomallei* in blood.[11] The lateral flow dipstick (LFD) is a detection method that can be read within 5-10 min by naked eyes as a point-of-care testing (POCT) and does not require special training. In 2014, Raymond L. et al. reported the prototype of melioidosis LFD assay using *B. pseudomallei* capsular polysaccharide (CPS) as an antigen for detection in clinical patient samples. These techniques specificity was as high as 97.2% (35/36) with the limit of detection comparable to ELISA at 0.2 ng/ml.[12] It gave low sensitivity, however, when clinical samples were used. Several PCR assays for detection of *B. pseudomallei* were developed which required 3-6 h. and the results have not been impressive for routine diagnosis.[11]

In this study, it was the aim therefore, to develop a PCR technique combined with the LFD (PCR-LFD) assay for the diagnosis of *B. pseudomallei*. The method could provide a higher sensitivity, specificity and the results can be observed on the LFD platform. The tests that were developed were also evaluated against clinical blood samples from patients suspected to be melioidosis and other infections.

Methods

Bacterial strains and culture conditions

All bacterial strains used for the spiking experiment in this study are listed in **Table 1**. Four clinical strains of *B. pseudomallei* were obtained from the Melioidosis Research Center (MRC), Khon Kaen University, Khon Kaen, Thailand. Two environmental and 6 clinical strains of other bacteria that were used as a negative control for the determination of the specificity of PCR assays were kindly provided by Asst. Prof. Dr. Umaporn Yordpratum, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

A single colony of each bacterium grown on Luria-Bertani medium (LB) agar was used to culture in 3 ml LB broth (Titan Biotech LTD., Rajasthan, India) with shaking at 200 rpm in a 37°C shaker incubator (Bio-active Co., Ltd, Bangkok, Thailand) overnight. Subsequently, 1% inoculums were inoculated into 5 ml of LB broth and cultured until they reached their mid-log phase and were used in the spiking experiment. The number of bacteria (CFU/ml) were quantitated by plate count on LB agar.

Clinical blood samples

Eighty-six left over EDTA blood samples were obtained from patients suspected to have bacterial sepsis. The septic patient blood samples were taken on the day of admission for hemoculture and a separate 1 ml for an EDTA tube for CBC and other tests. The left-over EDTA tubes were kept at 4°C until used but not more than 7 days. The hemoculture result from BD BACTEC™ blood culture media were then checked in all samples. Gram stains then were performed on positive blood culture broths, prior to subculture on blood agar, MacConkey agar and the bacteria were identified by standard biochemical tests.[13]

Table 1. Bacterial strains used in this study.

Bacterial strains	Sources
B. pseudomallei K96243	Patient's blood isolate, MRC, KKU
B. pseudomallei 844	Patient's blood isolate, MRC, KKU
B. pseudomallei 1909A	Patient's blood isolate, MRC, KKU
B. pseudomallei 1026b	Patient's blood isolate, MRC, KKU
B. thailandensis UE5	Environmental isolate from Thailand, MRC, KKU
B. thailandensis E264	Environmental isolate from Thailand, MRC, KKU
E. coli ATCC25922	ATCC
Salmonella group B	Patient's blood isolate, Khon Kaen Hospital
S. aureus	Kindly provided by Asst. Prof. Dr. Umaporn Yordpratum
P. aeruginosa	Kindly provided by Asst. Prof. Dr. Umaporn Yordpratum
K. pneumoniae	Kindly provided by Asst. Prof. Dr. Umaporn Yordpratum
S. epidermidis	Kindly provided by Asst. Prof. Dr. Umaporn Yordpratum

Patients who were culture positive for *B. pseudomallei* from clinical specimens were selected as tested cases. Patients with positive blood cultures for other bacteria or yeast were selected as controls. The EDTA blood samples were obtained from five healthy donors and were used as negative controls. To enhance the reliability of the PCR results, the PCR and PCR-LFD experiments were conducted and interpreted in a blinded manner. In this study, 43 cases cultured positive for *B. pseudomallei* and 43 cases were culture positive for other bacteria or yeast which were *Klebsiella pneumoniae*, *Enterococcus gallinarum*, *Enterococcus faecium*, *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Aeromonas hydrophila*, *Staphylococcus haemolyticus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus hominis*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Cryptococcus neoformans*, *Candida albicans*, *Aeromonas caviae*, *Enterococcus faecalis*, and *Klebsiella variicola*.

Blood samples were obtained during the period of June 2022 to January 2023. The protocol was approved by the Research Ethics Committee of Khon Kaen University and Khon Kaen Hospital, Thailand (HE641052 and KEMOU64030). The patient data that consisted of the specimen data collection, ages, culture confirmed dates, types of specimens and the culture laboratory results are listed in **Table 2**.

Table 2. The details of 43 patients with culture positive for *B. pseudomallei*.

No.	Age	Specimens	Sample dates	Culture confirmed dates	Microbial Agents
M01	66	Blood	5/6/2022	5/6/2022	*B. pseudomallei*
M02	62	Blood	3/6/2022	4/6/2022	*B. pseudomallei*
M03	62	Blood	6/6/2022	7/6/2022	*B. pseudomallei*
M04	33	Blood	10/6/2022	9-10/6/2022	*B. pseudomallei*
M05	62	Blood	13/6/2022	11/6/2022	*B. pseudomallei*
M06	31	Blood	11/6/2022	10/6/2022	*B. pseudomallei*
M07	59	Blood	12/6/2022	12/6/2022	*B. pseudomallei*
M08	44	Blood	20/6/2022	19/6/2022	*B. pseudomallei*
M09	52	Blood	21/6/2022	23/6/2022	*B. pseudomallei*
M10	72	Blood	20/6/2022	23/6/2022	*B. pseudomallei*
M11	50	Blood	20/6/2022	23/6/2022	*B. pseudomallei*
M12	89	Blood	13/6/2022	16/6/2022	*B. pseudomallei*
M13	53	Blood	18/6/2022	23/6/2022	*B. pseudomallei*
M14	48	Blood	19/6/2022	18/6/2022	*B. pseudomallei*
M15	47	Blood	15/6/2022	19/6/2022	*B. pseudomallei*
M16	53	Blood	18/6/2022	17/6/2022	*B. pseudomallei*
M17	54	Blood	22/6/2022	26/6/2022	*B. pseudomallei*
M18	37	Blood	25/6/2022	27/6/2022	*B. pseudomallei*
M19	48	Blood	26/6/2022	28/6/2022	*B. pseudomallei*
M20	76	Blood	27/6/2022	29/6/2022	*B. pseudomallei*
M21	50	Blood	29/6/2022	1/7/2022	*B. pseudomallei*
M22	51	Blood	30/6/2022	1/7/2022	*B. pseudomallei*

Table 2. The details of 43 patients with culture positive for *B. pseudomallei*. (Cont.)

No.	Age	Specimens	Sample dates	Culture confirmed dates	Microbial Agents
M23	53	Blood	3/7/2022	30/6/2022	*B. pseudomallei*
M24	47	Blood	6/7/2022	7/7/2022	*B. pseudomallei*
M25	56	Blood	7/7/2022	8/7/2022	*B. pseudomallei*
M26	35	Blood	11/7/2022	14/7/2022	*B. pseudomallei*
M27	70	Blood	13/7/2022	7/7/2022	*B. pseudomallei*
M28	55	Blood	14/7/2022	17/7/2022	*B. pseudomallei*
M29	51	Blood	15/7/2022	18/7/2022	*B. pseudomallei*
M30	55	Blood	16/7/2022	18/7/2022	*B. pseudomallei*
M31	53	Blood	26/7/2022	29/7/2022	*B. pseudomallei*
M32	44	Blood	27/7/2022	24/7/2022	*B. pseudomallei*
M33	80	Blood	27/7/2022	27/7/2022	*B. pseudomallei*
M34	51	Blood	27/7/2022	26/7/2022	*B. pseudomallei*
M35	54	Blood	26/7/2022	26/7/2022	*B. pseudomallei*
M36	75	Blood	26/7/2022	28/7/2022	*B. pseudomallei*
M37	60	Blood	26/7/2022	28/7/2022	*B. pseudomallei*
M38	65	Blood	26/7/2022	29/7/2022	*B. pseudomallei*
M39	47	Blood	27/7/2022	28/7/2022	*B. pseudomallei*
M40	59	Blood	27/7/2022	31/7/2022	*B. pseudomallei*
M41	51	Blood	28/7/2022	31/7/2022	*B. pseudomallei*
M42	61	Blood	28/7/2022	29/7/2022	*B. pseudomallei*
M43	64	Blood	30/7/2022	31/7/2022	*B. pseudomallei*

Table 2. The details of 43 cases who were positive with other bacterial infections.

No.	Age	Specimens	Sample dates	Culture confirmed dates	Microbial Agents
NM01	74	Blood	05/01/2023	08/01/2023	*K. pneumoniae*
NM02	51	Blood	05/01/2023	08/01/2023	*E. gallinarum*
NM03	69	Blood	05/01/2023	07/01/2023	*E. faecium*
NM04	65	Blood	05/01/2023	08/01/2023	*E. coli*
NM05	7	Blood	05/01/2023	10/01/2023	*S. aureus*
NM06	86	Blood	06/01/2023	08/01/2023	*E. coli*
NM07	25	Blood	06/01/2023	08/01/2023	*A. baumannii*
NM08	46	Blood	06/01/2023	08/01/2023	*A. hydrophila*
NM09	50	Blood	06/01/2023	09/01/2023	*E. coli*
NM10	72	Blood	06/01/2023	09/01/2023	*E. coli*
NM11	44	Blood	06/01/2023	09/01/2023	*S. haemolyticus*
NM12	86	Blood	06/01/2023	08/01/2023	*A. baumannii*

Table 2. (Continued)

No.	Age	Specimens	Sample dates	Culture confirmed dates	Microbial Agents
NM13	28	Blood	06/01/2023	08/01/2023	*S. aureus*
NM14	58	Blood	06/01/2023	08/01/2023	*S. pneumoniae*
NM15	74	Blood	06/01/2023	09/01/2023	*E. coli*
NM16	50	Blood	07/01/2023	09/01/2023	*P. aeruginosa*
NM17	73	Blood	07/01/2023	10/01/2023	*S. haemolyticus*
NM18	50	Blood	07/01/2023	10/01/2023	*E. cloacae*
NM19	78	Blood	07/01/2023	10/01/2023	*S. hominis*
NM20	64	Blood	07/01/2023	10/01/2023	*S. haemolyticus*
NM21	85	Blood	07/01/2023	10/01/2023	*S. haemolyticus*
NM22	46	Blood	07/01/2023	10/01/2023	*S. pneumoniae*

Table 2. The details of 43 cases who gave positive with other bacterial infections (Cont.)

No.	Age	Specimens	Sample dates	Culture confirmed dates	Microbial Agents
NM23	74	Blood	08/01/2023	10/01/2023	*E. coli*
NM24	40	Blood	08/01/2023	10/01/2023	*A. hydrophila*
NM25	72	Blood	08/01/2023	11/01/2023	*E. coli*
NM26	66	Blood	08/01/2023	12/01/2023	*K. pneumoniae*
NM27	80	Blood	08/01/2023	10/01/2023	*S. aureus*
NM28	84	Blood	08/01/2023	11/01/2023	*A. baumannii*
NM29	61	Blood	08/01/2023	12/01/2023	*K. pneumoniae*
NM30	72	Blood	09/01/2023	11/01/2023	*E. aerogenes*
NM31	67	Blood	09/01/2023	11/01/2023	*A. baumannii*
NM32	72	Blood	09/01/2023	12/01/2023	*K. pneumoniae*
NM33	72	Blood	09/01/2023	12/01/2023	*P. mirabilis*
NM34	50	Blood	09/01/2023	12/01/2023	*K. pneumoniae*
NM35	57	Blood	09/01/2023	12/01/2023	*S. aureus*
NM36	47	Blood	10/01/2023	12/01/2023	*K. pneumoniae*
NM37	47	Blood	09/01/2023	14/01/2023	*C. neoformans*
NM38	74	Blood	10/01/2023	13/01/2023	*A. baumannii*
NM39	72	Blood	10/01/2023	13/01/2023	*E. faecium*
NM40	54	Blood	11/01/2023	14/01/2023	*C. albicans*
NM41	56	Blood	11/01/2023	14/01/2023	*E. cloacae*
NM42	49	Blood	11/01/2023	14/01/2023	*A. caviae*
					E. cloacae
NM43	59	Blood	11/01/2023	14/01/2023	*E. faecalis*
					K. variicola

DNA preparation

Total cellular DNAs were extracted from each microbe by the method described by Anderson et al. with some modifications.[14] Briefly, 1.5 ml of an overnight bacterial culture in LB broth were centrifuged at 2,800 × g for 5 min (Beckman Microfuge, CA), resuspended the pellets with 380 µl of lysis buffer containing 1M Tris pH 8.0, 500 mM EDTA and 200 mM sucrose and then 5 µl of 20 mg/ml RNase were added (Thermo scientific, Carlsbad, CA). After being well mixed, twenty microliters of proteinase K (3 mg/ml), 100 µl of NaCl (5M) and 80 µl of CTAB/NaCl were then added to the mixture and incubated for 10 min at 60°C. The proteins were removed by extraction with an equal volume of phenol:chloroform (1:1) and centrifuged at 12,000 × g at 4°C for 15 min (Beckman Microfuge, CA). The aqueous phase was transferred to a new microcentrifuge tube and an equal volume of chilled 100% absolute ethanol was added to precipitate the DNAs and finally resuspended the DNAs in 50 µl of TE buffer. The DNA concentration was estimated by NanoDrop™ 2000/2000c spectrophotometer (Thermo-Fisher, Massachusetts, USA). Genomic DNA from each microbe was used for specificity and sensitivity validation of both PCR and PCR-LFD assays.

Preparation of blood samples for PCR and PCR-LFD assays

For sensitivity test, normal healthy human blood was spiked with bacterial cells. The mid-log *B. pseudomallei* K96243 culture was 10-fold serial diluted and the bacterial count was done on LB agar. Two hundred microliters of normal human EDTA blood was spiked with 10-fold serial dilution to get the bacterial numbers of 10^6 CFU to 1 CFU of *B. pseudomallei*. The 200 µl of spiked EDTA blood or 1 ml of EDTA patient's samples were centrifuged at 12,000 × g for 5 min and the supernatant discarded. The red blood cells in the pellet were lysed by being vortexed with 1 ml of sterile distilled water, and washed 3 times then centrifuged at 12,000 × g for 5 min. The pellet was resuspended in 20 µl of TE buffer before boiled at 100°C for 10 min in a heat block (Hercuvan, Malaysia) and 5 µl was subjected to PCR.

PCR Amplification

Specific primers for PCR amplification of *B. pseudomallei* were designed based on *wcbG* gene[15] from the genome sequence of *B. pseudomallei* strain K96243 (accession number NC_006350) using the primer-BLAST program. For PCR-LFD assay, the primers were designed to be biotinylated at the reverse primer (5′-CGGAGCGATCCAATGTTCA-3′) and FITC at the forward primer (5′-TGGCCGAATCGAGCG CGG-3′). These primer sequences were checked for their specificity by using the primer-BLAST program (https://www.ncbi.nlm.nih.gov/nuccore/NC_006350) against bacterial genome sequences in the NCBI database.

The PCR amplification was performed in a total volume of 25 µl using a PCR machine (Thermo Fisher Scientific Inc, Sweden). The reaction mixture consisted of 2.5 µl of 10× PCR buffer, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 1.5 mM of $MgCl_2$, 10% of DMSO, 0.2 µM of each primer, 0.1, 1 U Taq DNA polymerase and 5 µl of bacterial gDNA or processed blood samples. The optimal amplification condition started by initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 15 sec followed by final extension 7 min at 72°C (**Supplement figure 1**). The PCR amplification products were checked for their correct sizes on 2.5% agarose gel electrophoresis.

Samples subjected to PCR were various concentration of bacterial genomic DNA, various bacterial numbers spiked EDTA blood and patient's EDTA blood samples

Determination of sensitivity and specificity of the test

The detection limits of the PCR and PCR-LFD assays were performed using *B. pseudomallei* genomic DNA and a known amount of the bacterial cells. Ten-fold serially diluted bacterial genomic DNAs from 5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg and 5 fg were used for PCR. In addition, the 1-1 × 10^6 CFU spiking EDTA blood samples were also performed. For specificity, 5 ng of bacterial genomics DNA obtained from *B. pseudomallei* K96243, *B. pseudomallei* 844, *B. pseudomallei* 1909A, *B. pseudomallei* 1026b, *B. thailandensis* UE5, *B. thailandensis* E264, *S. aureus*, *E. coli* ATCC25922, *Salmonella* group B, *P. aeruginosa*, *K. pneumoniae* and *S. epidermidis* were used. Distilled water or normal healthy human blood were used as a negative control. All samples were performed in duplicated to determine the sensitivity and specificity of the PCR and PCR-LFD assays.

Validation of PCR-LFD

After PCR reactions, 5 µl of the amplified products were transferred in a new tube with 100 µl of running buffer. The LFD (Kestrel Bio Sciences Co. Ltd., Thailand) was immersed into the mixture and the results were observed within 10 min as positive when two lines were visible (a control line and a test line) or as negative when only the control line was visible.

Results

Sensitivity and specificity of PCR and PCR-LFD

The PCR (**Supplement figure 1**) and PCR-LFD were successfully developed and evaluated. The limit of detection of both PCR alone and PCR-LFD was 50 fg (**Figure 1**). These methods showed positive with all 4 selected *B. pseudomallei* clinical strains (**Figure 2**). Other bacterial DNAs (**Table 1**) used for evaluation of PCR and PCR-LFD specificity showed no cross-reactivity in both methods (**Figure 2**).

The PCR and PCR-LFD were further evaluated for their sensitivity of detection of *B. pseudomallei* in spiked human healthy EDTA blood. Both PCR and PCR-LFD assays could detect as few as 1 CFU of *B. pseudomallei* per 200 µl of spiked human blood (**Figure 3**).

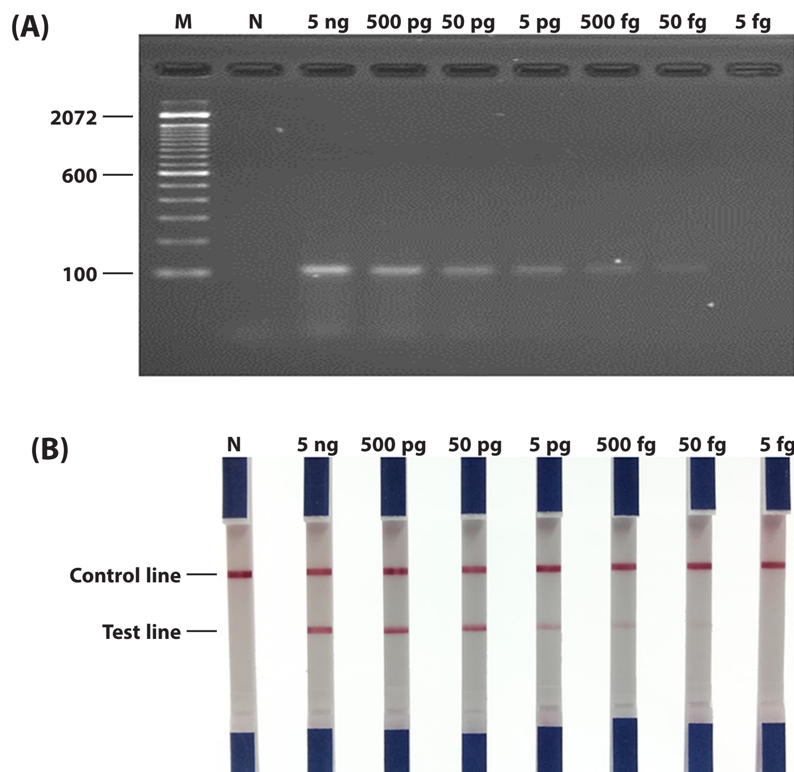

Figure 1. Sensitivity for detection of PCR and PCR-LFD.
Ethidium bromide-stained patterns of PCR products when *B. pseudomallei* genomic DNA from 5 ng to 5 fg were used as a template for amplification. Distilled water (DW) was used as a negative control (lane N). Lanes; M, 100 bp DNA Ladder. (B) PCR-LFD strips showed a positive band at the test line and control line when PCR products from amplification of *B. pseudomallei* DNA concentrations from 5 ng to 50 fg were further tested on PCR-LFD. Strip N is a negative control using DW.

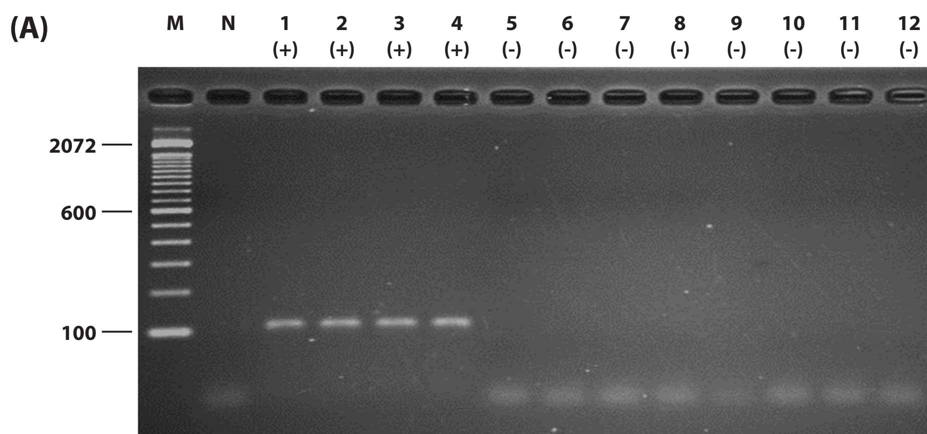

Figure 2. Specificity of PCR and PCR-LFD.
Ethidium bromide-stained patterns of PCR products from amplification of 5 ng of genomic DNA from *B. pseudomallei* K96243 (1), *B. pseudomallei* 844 (2), *B. pseudomallei* 1909A (3), *B. pseudomallei* 1026b (4), *B. thailandensis* UE5 (5), *B. thailandensis* E264 (6), *S. aureus* (7), *E. coli* ATCC25922 (8), *Salmonella* group B (9), *P. aeruginosa* (10), *K. pneumoniae* (11) and *S. epidermidis* (12). Lane N is a negative control and lane M is a 100 bp DNA ladder. (B) PCR-LFD assay results when each PCR product from (A) was reacted and flowed on LFD

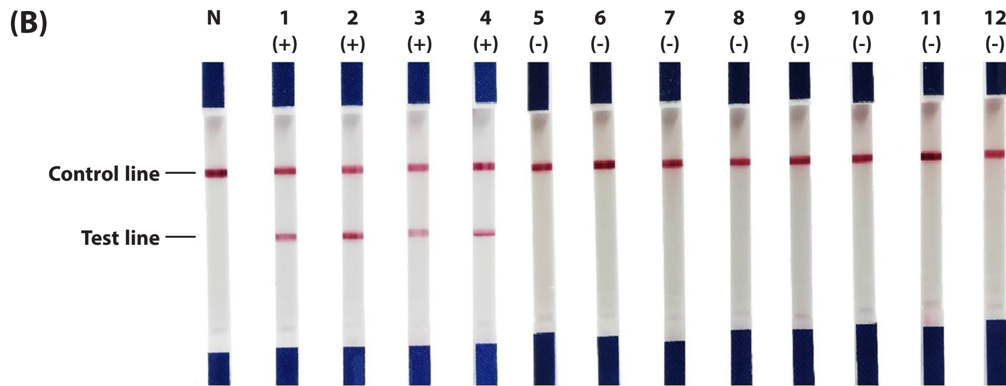

Figure 2. (Continued)

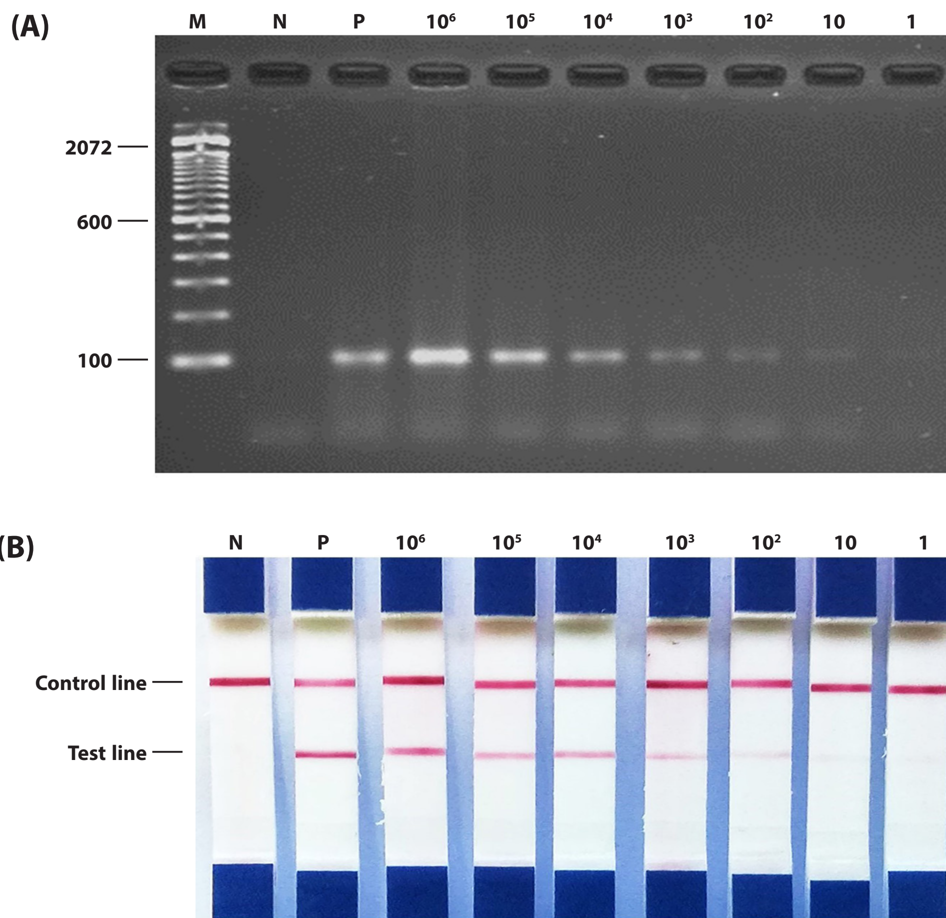

Figure 3. PCR and PCR-LFD detection of *B. pseudomallei* in spiked human blood samples.
Ethidium bromide-stained PCR products on agarose gel (A) and PCR-LFD results (B) were obtained from amplification of DNA extracted from 1 to 10^6 cells/ml viable bacterial cells that were spiked into EDTA blood (lanes 1-7 or strips 1-7). Lanes; M, 100 bp DNA ladder; N and P are negative and positive controls.

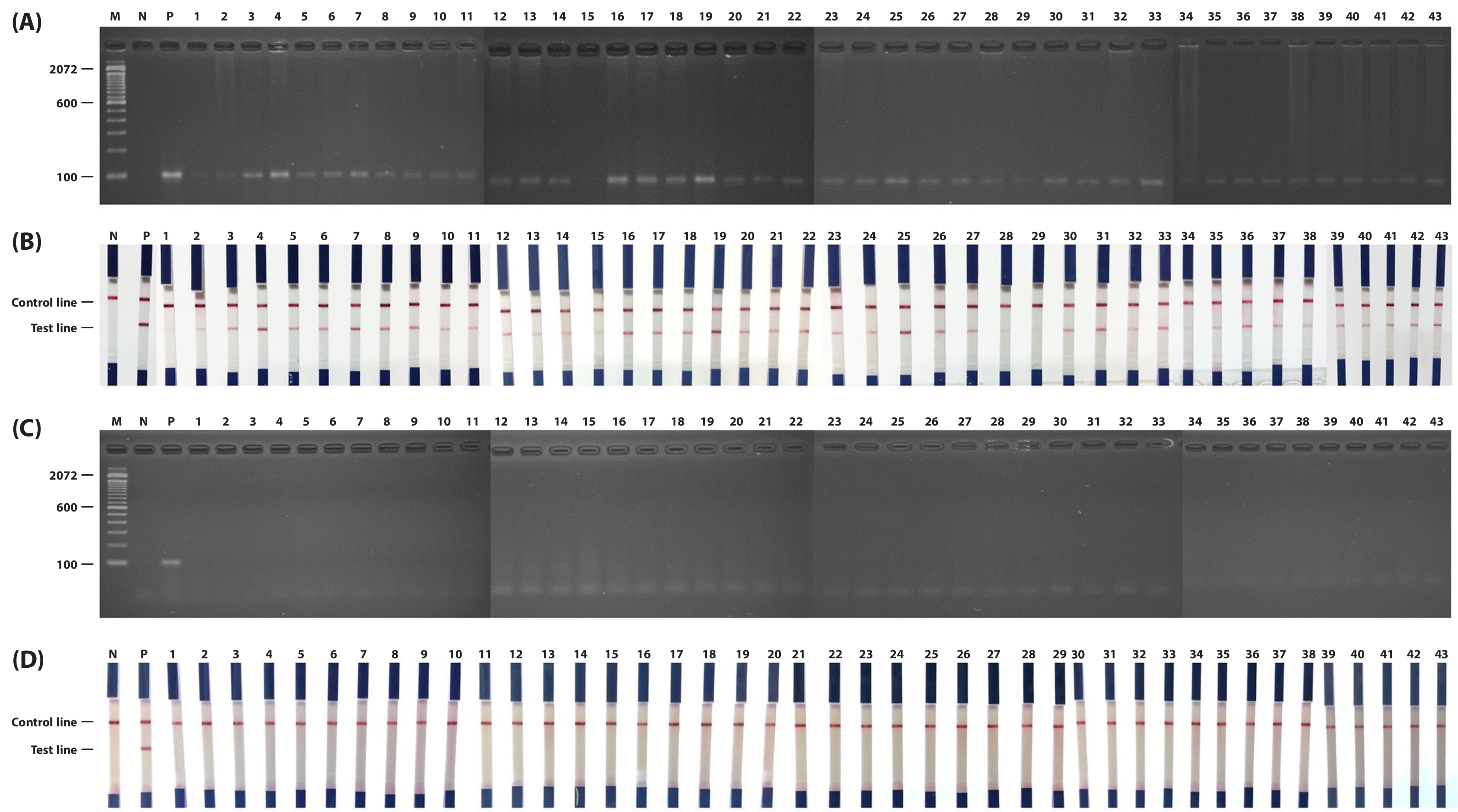

Figure 4. PCR and PCR-LFD detection of clinical blood samples.
Ethidium bromide-stained pattern of PCR products from 43 *B. pseudomallei* culture positive cases are shown in (A). Lane M was 100 bp DNA ladder, N and P are negative and positive controls. The PCR results shown in (C) were obtained from 43 culture-positive patients with infections caused by *K. pneumoniae* (1), *E. gallinarum* (2), *E. faecium* (3), *E. coli* (4, 6, 9 and 10), *S. aureus* (5), *A. baumannii* (7), *A. hydrophila* (8), *S. haemolyticus* (11), *A. baumannii* (12), *S. aureus* (13), *S. pneumoniae* (14 and 22), *E. coli* (15), *P. aeruginosa* (16), *S. haemolyticus* (17, 20 and 21), *E. cloacae* (18) and *S. hominis* (19), *E. coli* (23 and 25), *A. hydrophila* (24), *K. pneumoniae* (26, 29 and 32), *S. aureus* (27), *A. baumannii* (28 and 31), *E. aerogenes* (30), *P. mirabilis* (33), *K. pneumoniae* (34 and 36), *S. aureus* (35), *C. neoformans* (37), *A. baumannii* (38), *E. faecium* (39), *C. albicans* (40), *E. cloacae* (41), *A. caviae* (42), *E. cloacae*, *E. faecalis* and *K. variicola* (43). LFD strips 1-43 (B) are samples from 43 patients with *B. pseudomallei* culture positive and strips 1-43 (D) were blood samples that were positive for other microbes. N and P are positive and negative controls.

Detection of B. pseudomallei in clinical blood samples using PCR and PCR-LFD

When forty-three cases that were culture positive for *B. pseudomallei* and 43 cases that were positive for other bacteria and yeast were used to detect by PCR, all *B. pseudomallei* culture positive were positive (**Figure 4A**) while all 43 cases positive for other microbes were negative (**Figure 4C**). For PCR-LFD assays, all 43 *B. pseudomallei* cases were also positive (**Figure 4B**) while all 43-culture positives for other microbes, all showed negative results (**Figure 4D**).

Discussion

Bacterial hemoculture followed by identification using biochemical tests are still used as a gold standard for several bacterial infection identifications in human cases including melioidosis.[11,16] This method, however, requires 3 to 7 days for the processes of pre-enrichment or growth on selective culture medium.[4] Moreover, isolation of *B. pseudomallei* from non-sterile sites for example in fecal, pus or sputum specimens, can be difficult.[17,18] The direct detection of this bacterium in human blood can be difficult as a low number of bacterial (1-10 cells/ml) was found in the blood of infected patients.[19] This study successfully developed PCR and PCR-LFD with 100% sensitivity compared to the gold standard culture method as the EDTA-blood samples used were collected on the same day as hemoculture within the first 24 h of patient admission. The culture results were obtained 5-7 days after the date of blood collection when this proposed method could give results within 1.5 h. This indicated the usefulness of rapid diagnosis. Only whole blood samples were used in this study as it was aimed to focus on septic patients with a potential of high mortality where rapid diagnosis is essential. The EDTA blood samples were used rather than directly aliquoting from the hemoculture bottle to avoid dilution effect and the routine automate system. Our blood sample processing before PCR could eliminate most of anticoagulants.[20] Moreover, the leftover EDTA blood samples for not more than 7 days did not impacts the sensitivity results when compared with freshly blood.[14] You et al, demonstrated that blood from various leftover blood samples obtained from routine clinical tests are a good source of gDNA for research purposes.[21] The real-time-PCR was reported to be highly sensitive and specific for the diagnosis of melioidosis. Recently Noparatvarakorn et al.,[22] reported a type 3 secretion system 1 genes (TTS1) real-time PCR for detection of *B. pseudomallei* in clinical samples including blood, pus, sputum, urine, and peritoneal dialysis fluid. They showed that the method gave sensitivity and specificity of TTS1-PCR that were 78.2% and 100%. However, the lower sensitivity compared to ours might be due to different time and type of sample collections, targeted gene for primer design and sample processing. In addition, as both methods used PCR, the detection system is the main concern for sensitivity. Nevertheless, the real-time-PCR is quite difficult to set as a routine technique in general provincial hospitals due to the requirement of some skills, and the cost of the machines are quite high.

This current study aimed to develop a more rapid and sensitive DNA based method for the detection of *B. pseudomallei* in human blood that was easy to set up with a low cost. Primer sets for PCR detection and PCR-combined lateral flow dipstick assay (PCR-LFD) for diagnosis of melioidosis were designed. The specific primers used in this study were designed to target the *wcbG* gene on the bacterial chromosome I that was coded for a capsular polysaccharide biosynthesis protein localized in the bacterium cytoplasm. This gene was found only in pathogenic bacteria of Genus *Burkholderia* and was previously used for PCR detection.[14] These PCR and PCR-LFD assay results confirmed that this primer set developed by current authors was specific for *B. pseudomallei* and showed no cross reactions with other bacterial species that were tested (**Figure 2**). Although a limited number of bacterial strains were tested, most of them were commonly found in sepsis and this was then confirmed by the real clinical samples of various microbial infections. A previous primer set designed to target the *wcbG* gene showed the limit of detection as 32 fg of DNA (about 4 genomes) by qPCR for detection in soil samples[23] while this PCR-LFD method gave similar sensitivity to detect 50 fg of bacterial genomic DNA or 1 CFU/200 µl of blood samples. This PCR test is similar to previously published data by current authors that demonstrated that LPS1 and LPS2 primers could detect as little as 1 CFU/ml of *B. pseudomallei* in clinical blood samples.[20] This previously reported method, however, needed at least 1 ml of clinical samples and a lysis protocol was used to get genomic DNAs instead of 200 µl and DNA extraction that was essential in this method. A lateral flow immunoassay (LFI) for melioidosis was developed using monoclonal antibodies to capsular polysaccharide (CPS),[12] similar to the capsular polysaccharide biosynthesis protein *wcbG* gene in currently used PCR primers. Its sensitivity was comparable to the antigen-capture immunoassay (0.2 ng/ml). It could also detect antigens in clinical samples in either serum or urine and accept multiple sample matrices. Although LFI was very useful as a POCT in rural areas and using the rapid test as it is inexpensive and stable at room temperature, it gave low sensitivity in serum and urine in patient samples.[24–26] LFI alone therefore might not be sensitive enough for rapid screening. PCR tests are certainly sensitive, picking up tiny traces of bacterial material, but PCR does not necessarily mean that someone is infected. It may stay positive after the infection has gone as bacterial DNA or dead cells were detected. Rapid antigen lateral flow tests are developed to look for high bacterial load and thus may indicate people who are most likely to be infectious.

Current PCR in this laboratory used whole blood samples instead of serum in order to detect more bacterial materials residing in infected cells thus increasing the sensitivity. The combined PCR with LFD speeds up the detection time. Though this laboratory's PCR and PCR-LFD gave similar sensitivity, PCR-LFD was 100 times more sensitive, rapid and accurate than PCR alone as shown in *Bacillus anthracis*.[27] Moreover, PCR-LFD also gave

high sensitivity with 0.0013 ng/μl in detection of pure donkey meat in food samples. They showed that the assay gave about 10 times higher sensitivity than gel electrophoresis.[28]

Other key parameters of the success of the PCR-LFD assay included the ratio of primers (forward and reverse), concentration of each reagent, PCR reaction in temperatures and times, method for DNA extraction from clinical samples. As the DNA amplification was performed from whole blood, the bacterial DNA in serum or infected cells will not be excluded. Moreover, the strip test in this study found to be stable for 1-2 years at room temperature (25°C). When comparing the storage conditions of other strip tests, long term storage condition found to be stable for at least 1 year at room temperature. Nevertheless, the shelf life of the strip test depends on container, type of strip test, antibodies, label type and the storage conditions such as if the test strip container is opened, it is expired 180 days after opening or short-term exposure to 37°C might be reduces sensitivity of the test kits.[29] Nowadays, most of the hospitals have DNA extraction machines making this PCR-LFD method useful and simple for routine use. The DNA extraction technique with a greater yield would aid in the rapid diagnostics.[30,31] As the amplified DNA fragment was very small, allowing very efficient amplification and the use of 40 cycle permits more than 10^{12}-fold amplification under optimal conditions.[14,32] When comparing time used for detection of *B. pseudomallei*, PCR-LFD gave shortest time as only 1.5 h. compared to 3-7 days of culture and 2.10 h. of PCR alone before the result was obtained. Detection of bacterial DNA using PCR combined with lateral flow in this study increased sensitivity and speeded up the time for detection leading to future field or POCT testing.

Conclusion

In conclusion, this demonstrated PCR-LFD assay provided a rapid and simple method to detect of *B. pseudomallei* in whole blood samples. Although it is not a simple POCT test for routine use as only LFD alone, it was more sensitive reflect that DNA was extracted from EDTA blood, not use directly.

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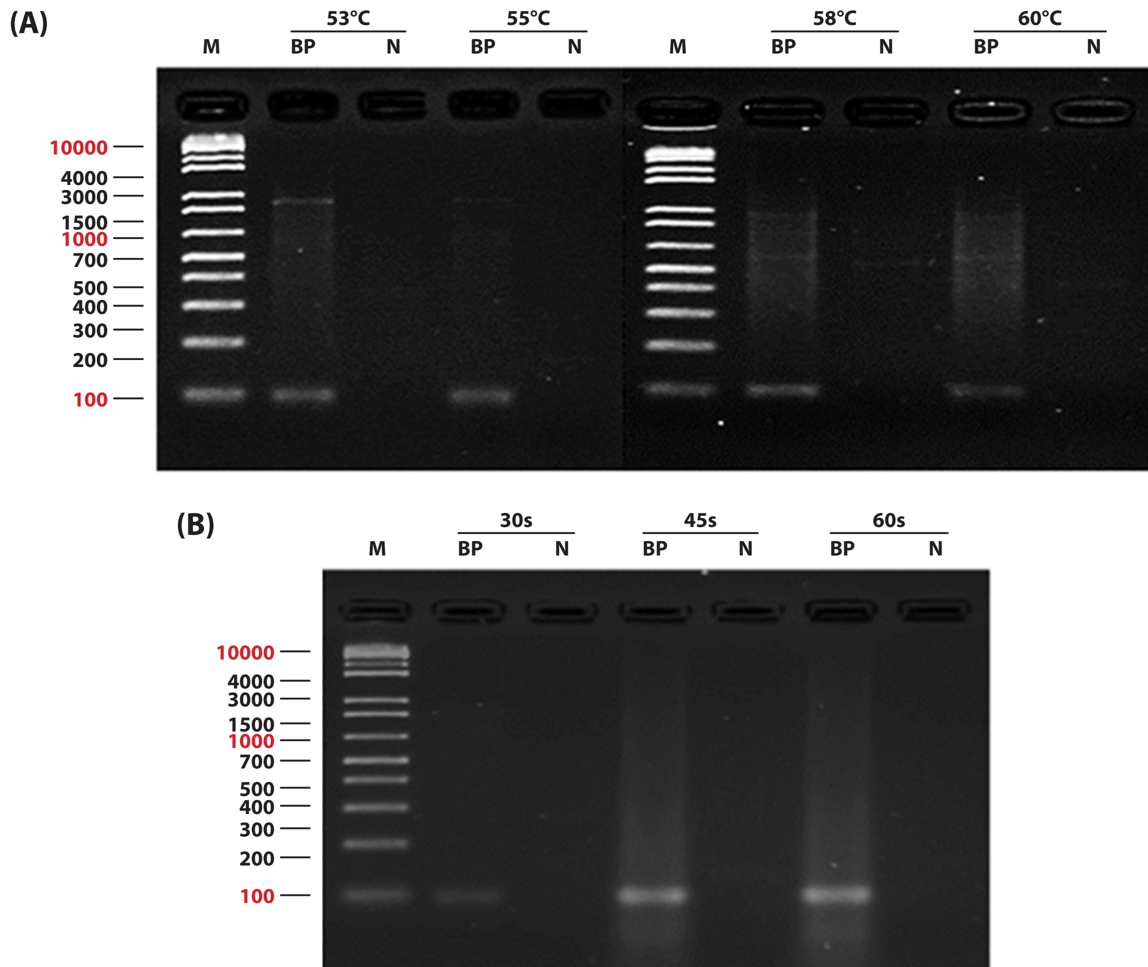
Supplement information



Supplementary Figure 1. Optimization of annealing temperatures.

Ethidium bromide-stained patterns of PCR products for the optimization of annealing temperatures (A) and times (B). Two microliters of DNA (5 ng) (BP) or DW as positive (P) and negative (N) controls were used for PCR using *wcbG* gene primers of *B. pseudomallei*. The reactions were run with various annealing temperatures starting from 53°C, 55°C, 58°C and 60°C and various annealing times from 30s to 60s. The PCR was run for 40 cycles and the products were then run on 2.5% agarose gel electrophoresis. Lanes; M, Invitrogen 1 Kb Plus DNA Ladder.