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Development of an immunomagnetic separation-ELISA for the detection of *Burkholderia pseudomallei* in blood samples

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Abstract

Background: Septicemic melioidosis caused by *Burkholderia pseudomallei* is a serious cause of morbidity and mortality. An effective, rapid and simple diagnostic method is required for detection of *B. pseudomallei* infection.

Objective: To develop immunomagnetic beads (IMB) coupled with ELISA (IMB-ELISA) for detection of *B. pseudomallei* in blood samples of patients with suspected melioidosis.

Methods: For separation of *B. pseudomallei* from buffer, blood samples and hemoculture, 200 nm immunomagnetic beads (IMBs) coated with 4B11 monoclonal antibody (4B11-IMBs) against exopolysaccharide antigens were used. The detection was done by an ELISA based biotin-streptavidin system. The sensitivity and specificity were evaluated.

Results: 4B11-IMBs (100 μg) were successfully developed and used for detection of *B. pseudomallei* in 1 ml samples. Transmission electron microscopy (TEM) imaging demonstrated *B. pseudomallei* was captured by 4B11-IMBs. The IMBs showed high capture efficiency (98%) with *B. pseudomallei* in buffer. The IMB-ELISA assay was highly specific for *B. pseudomallei*. It showed no cross-reactions with other bacteria, except *B. mallei*. The limits of the *B. pseudomallei* assay detection for detecting *B. pseudomallei* in either buffer solution or blood was 10^2 CFU/ml. The IMB-ELISA detection sensitivity in blood samples was 44.5%. Although it did not give the highest sensitivity, it was useful for detection with hemoculture that was faster than conventional methods.

Conclusion: This study suggests the IMB-ELISA assay offers a simple and highly specific method with a turnaround time of 6 h for detection of *B. pseudomallei*. The developed assay can be applied in hospitals for surveillance of *B. pseudomallei*.

Keywords: IMB-ELISA, *Burkholderia pseudomallei*, melioidosis, blood samples, diagnosis

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Introduction

Melioidosis is a severe disease caused by *Burkholderia pseudomallei*, which is endemic in Southeast Asia and Northern Australia. It infects via inoculation into wounds, ingestion or inhalation of soil or contaminated water. Northeastern Thailand has reported the highest prevalence.[1] The diagnosis of melioidosis is difficult due to a variety of signs and symptoms. The laboratory diagnosis of melioidosis still relies on bacterial isolation and identification by biochemical tests using the culture-based method as the gold standard that takes 3 to 7 days,[2] leading to patient death in some cases. Therefore, a rapid, sensitive, specific and simple diagnostic test is required for detection of the bacterial target, especially in blood samples, for early effective treatment. Many methods have been developed for rapid detection, including antibody, antigen and molecular based methods.

Indirect hemagglutination (IHA) is the most commonly used method but is not suitable for diagnosis of suspected acute melioidosis in patients in the endemic area. This is because of the high antibody background.[3] Enzyme-linked immunosorbent assay (ELISA) and related serodiagnostic strategies have been developed for increasing the accuracy for diagnosis using various antigens. The detection of *B. pseudomallei* antigens in

the endemic area have been reported using immunofluorescence assay (IFA) and latex agglutination (LA). The IFA based microscopic assay[4] can be done in 10 minutes and has been applied for rapid detection of *B. pseudomallei* in clinical samples. IFA is not suitable for detection of *B. pseudomallei* directly in blood samples because of low numbers of bacteria.[5] Therefore, it is recommended for detection in the endemic area using an automated blood culture system.[6] Although MAb-IFA is more rapid than the gold standard method, this assay needs a well-trained person and fluorescence microscope. The LA test is an effective, simple, and inexpensive technique that does not require special instruments for detection of *B. pseudomallei* in clinical samples. The LA assay cannot be used directly in blood samples but needs hemoculture processing before detection.[7] Lateral flow immunoassay (LFI) has been developed for detection of the capsular polysaccharide (CPS) of *B. pseudomallei* in clinical samples of patients with active melioidosis using serum, urine, sputum and blood.[8] The LFI prototype has a limit of detection (LOD) of 0.2 ng/ml and cannot detect the bacterial target in blood because of low amounts of CPS shedding and bacterial loads in the blood samples.[8]

The immunomagnetic bead (IMB) separation technique is an effective, rapid, specific and simple method that can selectively and specifically bind with the target cells in the sample matrix. The IMB separation technique can be used to concentrate bacterial cells and eliminate unwanted components and substances.[9] It has been applied to detect targets in the clinical samples.[10-12] The IMB technique can be successfully designed by using monoclonal antibodies (MAbs) or polyclonal antibodies (PAbs). Many antibodies can be used with this immunologically based method in combination with cultivation or biochemical identification for diagnosis. The combination of IMB with ELISA, or IMB-ELISA method, has been applied for detection of several bacteria, such as *Salmonella* in milk[13] and *Alicyclobacillus* spp. in apple juice.[14] The turnaround time using the IMB-ELISA method is more rapid than the conventional ELISA method.[15]

In this study, the IMB-ELISA technique was developed using the monoclonal antibody 4B11 for detection of *B. pseudomallei* in blood samples of melioidosis patients suspected of having septicemia. The monoclonal antibody 4B11 against the 200-kDa protein exopolysaccharide (EPS) of *B. pseudomallei* was successfully used for detection of *B. pseudomallei*.[5-7,16-18]

Methods

Blood samples

Twenty EDTA blood samples (1 ml per sample) were obtained from patients who were admitted to the Srinagarind and Khon Kaen Provincial Hospitals. During the period from February 2017 to August 2017, 16 samples were from suspected blood stream infections of *B. pseudomallei* (BSIs). In addition, there were four samples suspected to be infected with *B. pseudomallei* from an epidural abscess, sputum (2) and pus from a wound. The EDTA blood samples were obtained on the same or nearest day of admission and sent for the hemoculture procedure. The EDTA blood samples from healthy donors were used as the negative controls. The protocol was approved by the Human Research Ethics Committee of Khon Kaen University and Khon Kaen Hospitals, Thailand (HE591440 and KE60065). All samples were taken from left-over specimens; thus, the gathering of consents was not possible.

Antibodies and magnetic bead particles

The 4B11 monoclonal antibody (isotype IgG2a) against the 200-kDa EPS presented on the surface of all *B. pseudomallei* isolates was used.[18,19] The 4B11 hybridoma cells were kindly provided by Prof. Stitaya Sirisinha, Mahidol University, Bangkok, Thailand. Polyclonal antibodies derived from rabbit antibodies against whole *B. pseudomallei* cells (crude extracted antigen of *B. pseudomallei*) were raised and used throughout this study. The animal protocol was approved by the Animal Research Ethics Committee of Khon Kaen University (AEKKU 15/2555). Four different kinds of magnetic bead particles, including SiMAG-Cyanuric (C500) with the size of 500 nm, FluidMAG-Amine (F200) with the size of 200 nm and 2 sizes of SiMAG-Amine S500 (500 nm) and S750 (750 nm), were used in this study. All magnetic beads that were synthesized from Fe_3O_4 or iron oxide (ferric oxide) were purchased from Chemicell GmbH, Germany. The magnetic bead set-up and optimization process showed that F200 (200 nm) was the best bead for detection of *B. pseudomallei* and only the F200 beads were used throughout this study.

Coating of antibody on fluid-amine beads

1. 4B11 monoclonal antibody preparation

The hybridoma cells were grew in RPMI medium supplemented with 20% FBS, 0.4% 10 mg gentamicin, and 0.5% 0.01 M mercaptoethanol in IMDM and cultured in 5% CO_2. The supernatants containing the antibody were concentrated and purified by ammonium sulfate and desalted and purified by HiTrap™ Desalting and HiTrap™ Protein G column (GE Healthcare, UK).

2. Antibody coated on magnetic beads for producing 4B11-IMB

The F200 beads were coated to produce IMB by the covalent coupling procedure (carbodiimide method), according to the manufacturer's instructions with some modifications.[20] In this study, 4B11-IMB and uncoated-IMB represent the beads coated with 4B11 Mab and uncoated beads. Firstly, 1 mg F200 particles were washed twice with 1 ml MES buffer (0.1 M 2-(N-Morpholino) ethanesulfonic acid, pH 6.0) (Merck, Germany) prior to re-suspension in 0.25 ml MES buffer containing 10 mg fresh EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (Pierce, USA). The monoclonal antibodies at 0, 10, 50, 100, 120 μg/ml were added for titration of concentrations to coat the beads and mixed for 2 h. Bovine serum albumin (BSA) (2%) in PBS (0.01 M pH 7.4) with 0.05% NaN_3 was added for protection against non-specific reactions. The concentration of BSA (0.1%–8%) in the blocking buffer was optimized before use. The optimal concentration of BSA for blocking giving lowest background was 2% for 30 minutes. The particles were washed 3 times and stored at 4°C until used. The efficiency of bead coating was determined by direct detection of antibody on beads by ELISA and measurement of unbound antibody in the buffer by NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific Inc., Wilmington, USA). The capture efficiency (CE) of 4B11-IMB against *B. pseudomallei* was evaluated using the

formula as follows: CE = (1- B/A) × 100%), where A is the total number of cells present in the sample (CFU/ml) and B is the number of cells unbound to IMBs (CFU/ml) in the supernatant and wash solutions.[25] Then, 100 μg 4B11-IMB was added in 1 ml PBS containing *B. pseudomallei* at 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/ml to capture cells. The uncoated-IMB captured *B. pseudomallei* at 10^5 CFU/ml acting as a negative control. The irrelevant antibody coated-IMBs were also used as a negative control and a low background was obtained. The total number of *B. pseudomallei* were enumerated before capture by 4B11-IMB and after 1 h incubation. The unbound bacterial cells in the supernatant and washed buffer were enumerated by the drop plate assay method (incubation of NA plates at 37°C for 18 h). All experiments were performed in triplicate. The binding between 4B11-IMB and *B. pseudomallei* was confirmed by using transmission electron microscopy (TEM) (FEI, TECNAI G^2, Hillsboro, Oregon, USA).

IMB-ELISA in buffer and spiking blood samples

To develop a specific, sensitive and suitable IMB-ELISA, various substances in the ELISA assay were titrated against 10^5 CFU/ml *B. pseudomallei* cells, including dilutions of PAbs at 1:250 to 1:6000 as the detection antibodies, prior to being detected by biotinylated anti-rabbit IgG (Goat anti-Rabbit IgG (H+L) (Thermo Fisher Scientific Inc., Rockford, USA) at 1:10,000 to 1:50,000, and streptavidin-HRP (Streptavidin, Horseradish Peroxidase Conjugate, Merck KGaA, Darmstadt, Germany) at 1:4,000 to 1:20,000. The optimal ratio of PAb was 1:3,000 and 1:12,000 for biotinylated anti-rabbit IgG and 1:10,000 for streptavidin-HRP.

The IMB-ELISA was tested for detection of *B. pseudomallei* in buffer and spiked blood samples. *B. pseudomallei* was diluted to 10^5 CFU/ml in 1 ml PBS; meanwhile, the fresh whole blood obtained from a registered blood bank was spiked. A schematic illustration of the system for detection of *B. pseudomallei* by IMB-ELISA is shown in **Figure 1**. The 100 μg 4B11-IMBs were added into buffer in microtubes containing of *B. pseudomallei* then mixed for 1 h. The 4B11-IMB bacterial complexes were captured and precipitated at the sides of the microtubes when placed on a magnetic separator for 5 minutes and the supernatants were removed. The reaction tubes were washed and 200 μl 1:3,000 PAb was added in the reaction tubes and incubated for 30 minutes. The supernatants that contained unbound PAb were removed and washed, and 200 μl 1:12,000 biotinylated anti-rabbit IgG was added and incubated for 30 minutes. The unbound conjugate was removed and this was followed by washing 3 times, then 200 μl of 1:10,000 streptavidin-HRP in 2% BSA in 0.01 M pH 7.4 PBS with 0.05% Tween 20 was added, mixed and incubated for 30 minutes at room temperature (RT). After washing, 100 μl OPD (1,2-phenylenediamine dihydrochloride; Dako) substrate was added. The end product was transferred into a 96 well plate (Nunc, Roskilde,

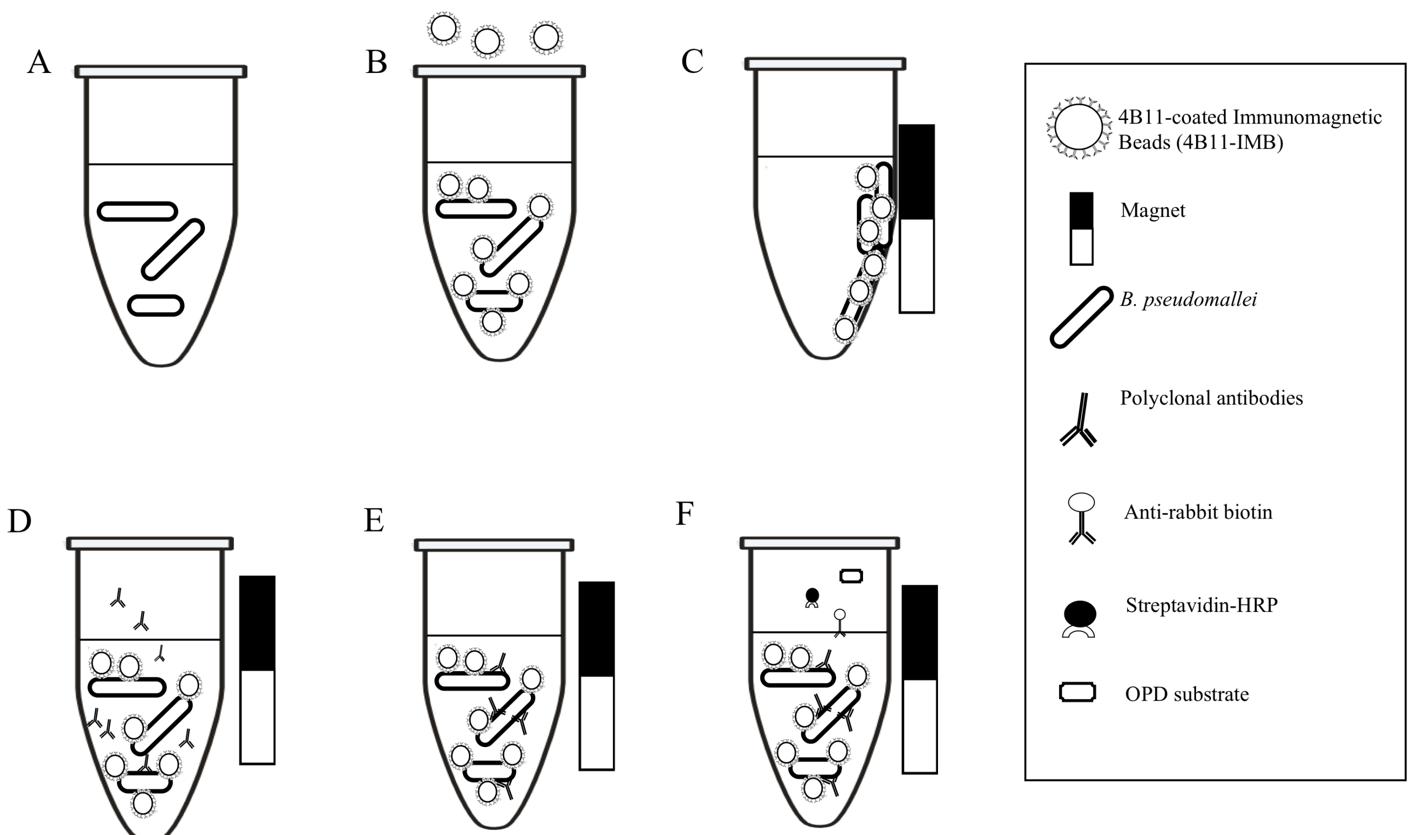

Figure 1. Schematic illustration of the system for detection of *Burkholderia pseudomallei* by IMB-ELISA. Bacteria in 1 ml of blood samples in microtubes (A) were captured by 4B11-IMB (B). After incubation for 1 h, the bacteria-bound beads were separated by magnetic force (C) and the unbound substances were removed from the reaction. The secondary (PAb) against *B. pseudomallei* were then added and incubated for 30 min (D) followed by removing the excess antibodies (E). The conjugated antibodies (biotinylated anti-rabbit antibodies and streptavidin-HRP) were added (F) for detection by ELISA.

Denmark), and the absorbance measurement was carried out at 492 nm with the ELISA reader (TECAN Infinite 200, Tecan Group Ltd, San Jose, USA).

IMB-ELISA for detection of B. pseudomallei in hemoculture

B. pseudomallei at concentrations of 10^3, 10^2, and 10^1 CFU/ml were spiked into 5 ml fresh blood and then injected into blood culture bottles that contained 45 ml of hemoculture media (Liquid broth, Himedia, India). The hemoculture bottles were incubated at 37°C. The tests were performed in triplicate for each bacterial concentration. After culture for 0, 5, 10, 15, 24 and 48 h, 100 µl of each sample from the hemoculture bottles were taken for either culture with biochemical identification or with IMB-ELISA for comparison. The times required for positive detection and identification were then compared.

Specificity and sensitivity of IMB-ELISA

For the specificity testing, *S. aureus*, *E. coli*, *B. mallei*, *B. cepacia*, *B. thailandensis* and *P. aeruginosa* were cultured in LB at 37°C to mid-log phase and diluted to 10^5 CFU/ml in buffer. For the sensitivity testing, *B. pseudomallei* were serially diluted in PBS buffer from 10^0 to 10^7 CFU/ml and used to determine the sensitivity of IMB-ELISA in buffer and spiked blood samples. The bacterial dilutions of 100 µl containing concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 CFU/ml were placed into 900 µl of buffer and fresh EDTA blood samples of healthy persons. The detection procedure was the same as the IMB -ELISA.

Sample tests

The samples of EDTA blood (1 ml) were tested by using the same procedures as with IMB-ELISA in the buffer and blood samples as previously mentioned.

Statistical analysis

The statistical methods that were used in this study included the t-test, paired t-test, and one-way analysis of variance (ANOVA) for comparisons. Data were analyzed by using the IMB® SPSS® version 23. All data were presented as mean values with their standard deviations (mean ± SD). The cut-off line of IMB-ELISA could be calculated by using the mean of absorbance of the negative control plus three SD (Background Cut-off = Mean of Negatives + [3SD of Negatives]). The OD of the negative control was obtained from detection of 4B11-IMB in the blood without bacteria. Therefore, the cut-off value was 0.52.

Results

Optimization and efficiency of antibody for producing 4B11-IMB

The Fluid-MAG (F200) showed an optimum value when used with the ELISA for detection of *B. pseudomallei*. The concentrations of MAb coated on beads were detected by ELISA and the results are shown in **Figure 2A**. Results demonstrated that the optimal concentrations of MAb coated on the beads were 100 mg/ml and 120 mg/ml; however, only the MAb at 120 mg/ml was selected. The coating efficiency was determined and the results shown in **Figure 2B** demonstrate that most antibodies used for coating are significantly bound on the beads. The results demonstrated that the optimal 4B11-IMB concentrations could bind nearly 100% of all bacteria (98% of CE at 3 × 10^2 cells, **Figure 2C**). In contrast, when the higher numbers of bacteria were used, lower efficiency was obtained. The 4B11-IMB-bound *B. pseudomallei* in buffer and the negative control beads are shown in the TEM photograph in **Figures 2D and 2E**, respectively.

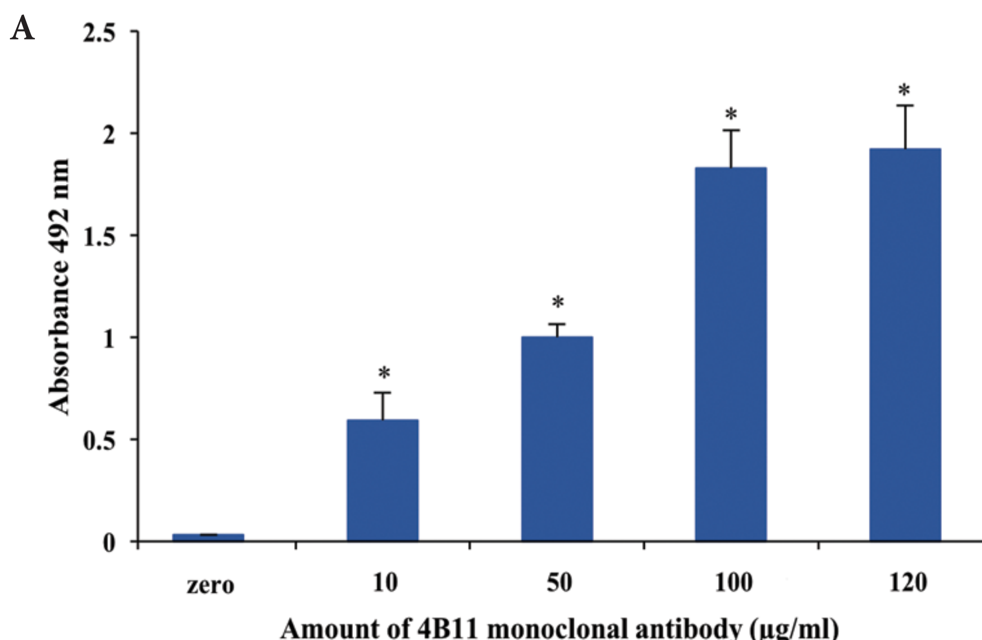

Figure 2. The optimization of coating antibodies for MAb-coated IMB. The various concentrations of 0, 10, 50, 100 and 120 mg/ml of monoclonal antibody 4B11 were coated on beads and concentrations of bound antibodies were determined by ELISA (A).

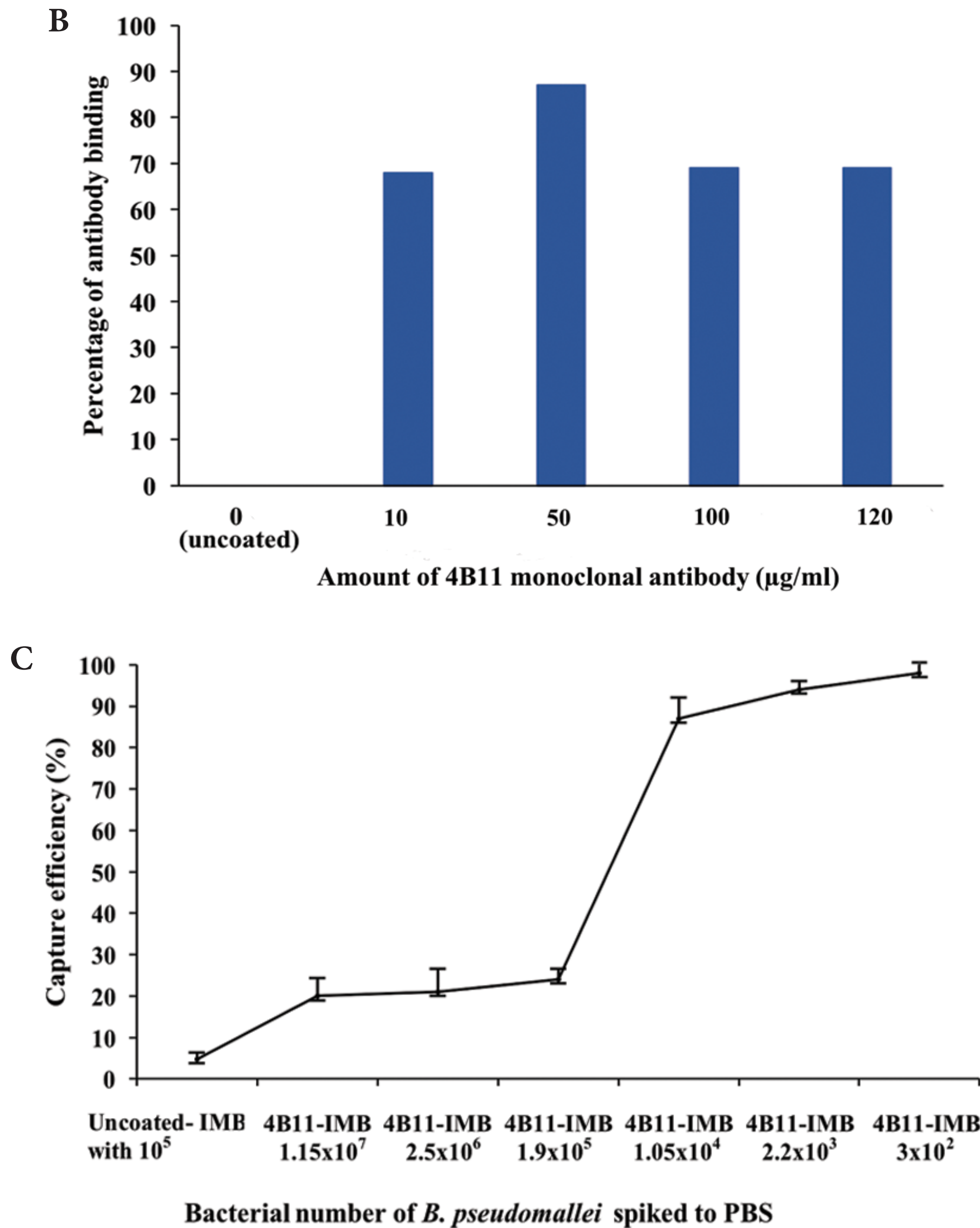

Figure 2. (Continued) Each bar represents mean ± SD of the absorbance of antibody coated on the beads. The asterisks (*) indicate statistical significance ($p < 0.05$) compare to control. The percentage of antibody binding was calculated by the percent of the amount of antibody added minus the amount of antibody left divided by the amount of antibody added (B). The optimal coated-IMB (4B11-IMB) were evaluated for capture efficiency using 3×10^2, 2.2×10^3, 1.5×10^4, 1.9×10^5, 2.5×10^6, 1.5×10^7 *B. pseudomallei* cells (C).

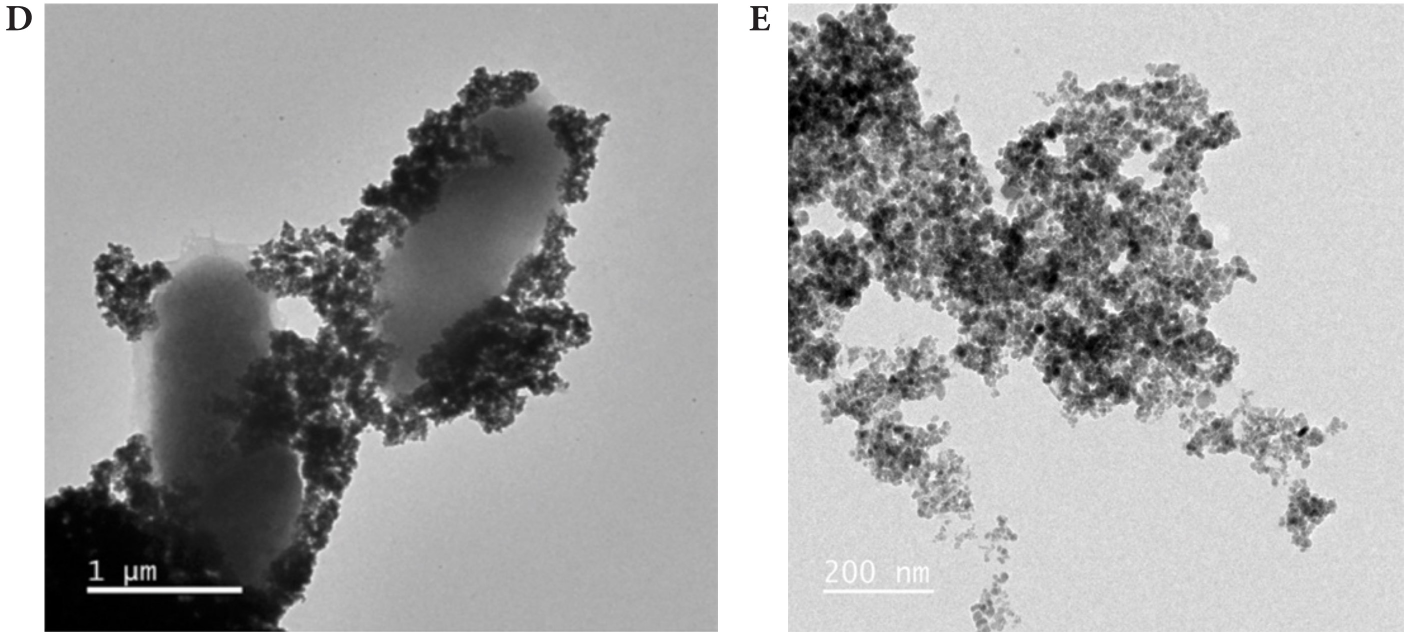

Figure 2. (Continued) The bound and unbound bacteria were measured by the culture method and the percentage of capture efficiency (CE) calculated to evaluate the ability of the bead for binding, capture and separation of bacteria. The line represents the mean of CE ± SD. The uncoated-IMB used for capturing *B. pseudomallei* at 10^5 CFU/ml was used as a negative control. TEM image of the F200 IMB-bound *B. pseudomallei* in buffer (PBS) (D). The negative control beads are shown (E).

Specificity and sensitivity of IMB-ELISA

For specificity testing, various bacteria including *B. pseudomallei*, *P. aeruginosa*, *B. mallei*, *B. cepacia*, *B. thailandensis*, *E. coli* and *S. aureus* were placed at 10^5 CFU/ml into 1 ml of buffer and then detected by IMB-ELISA. The results demonstrated that this method gave a negative response in all other bacteria tested with high specificity for *B. pseudomallei* and *B. mallei* (**Figure 3A**), although *E. coli* gave more background than others. Our result demonstrated that the bacteria at 10^2 CFU/mL was the lowest dilution that registered an OD > 0.52. The sensitivity for detection in the buffer and spiked blood samples was 10^2 CFU/ml (**Figure 3B**).

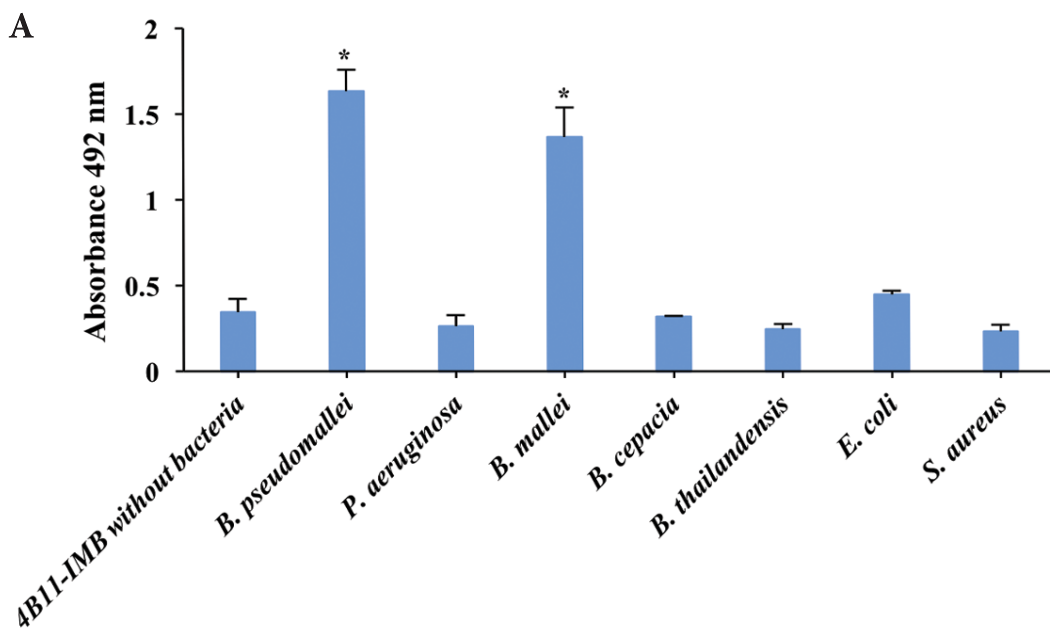

Figure 3. Specificity and sensitivity of IMB-ELISA for detection of *B. pseudomallei*. For the specificity test, 10^5 CFU/ml of various bacteria were spiked into 1ml EDTA blood and detected by IMB-ELISA while the 4B11-IMB without bacteria was used as the negative control (A).

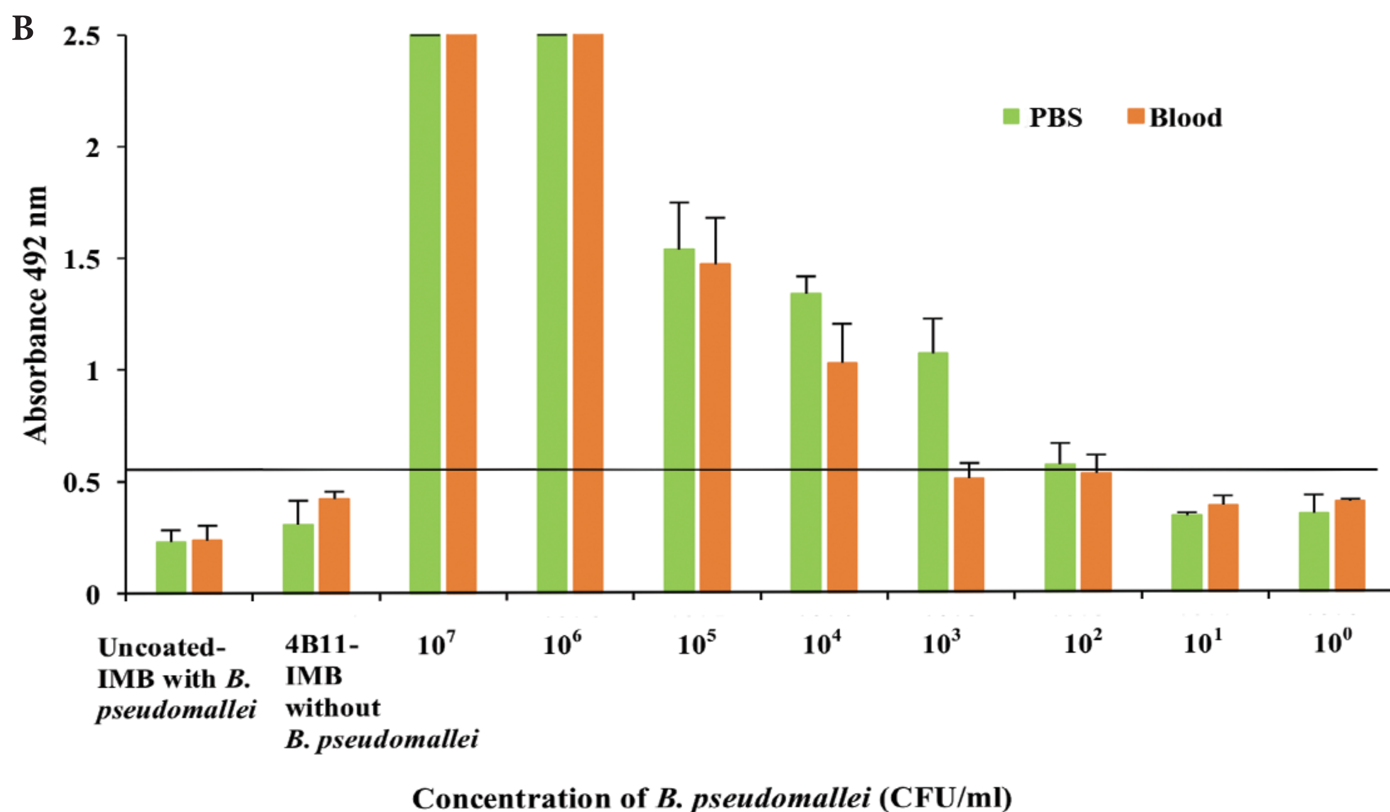

Figure 3. (Continued) Each bar represents mean ± SD. The asterisks (*) indicate statistical significance ($p < 0.05$) compare to control. Sensitivity of IMB-ELISA was tested for detection of *B. pseudomallei* in buffer compared with inoculated blood samples (B). The *B. pseudomallei* was 10-fold serially diluted from 10^0 to 10^7 CFU/ml into 1 ml of PBS buffer and EDTA blood prior for detection by using IMB-ELISA. The negative control groups included uncoated-IMB with 10^5 CFU/ml of *B. pseudomallei* and 4B11-IMB without bacteria. The absorbance by the ELISA reader was at 492 nm. Each bar represents mean ± SD. The line represents the cut off (Mean of 4B11-IMB without bacteria plus 3 SD) of the IMB-ELISA test.

Detection of B. pseudomallei in blood samples

Thirteen 1 ml EDTA blood samples were tested by IMB-ELISA. The samples were positive in 4/20 samples for IMB-ELISA (**Table 1**). Out of 20 samples, 16 samples were suspected blood stream infections (BSIs) with *B. pseudomallei* and four samples were infected with *B. pseudomallei* from other sources. A total of 9 out of 16 of the BSIs were confirmed positive by hemoculture; 7 were negative by hemoculture. There were four positives with IMB-ELISA, which correlated with hemoculture positive results. Therefore, the sensitivity of IMB-ELISA was 44.5% (4/9) when comparing positive cultures.

Table 1. Detection of *B. pseudomallei* in the blood samples of suspected melioidosis patients by hemoculture and IMB-ELISA

Sample number	Specimen sites*	Method for detections		Detection date	
		Hemoculture	IMB-ELISA/OD value	Hemoculture	IMB-ELISA
1	Blood	+	+/0.623	28-Feb-17	2-Mar-17
2	Blood	+	-0.407	12-Mar-17	14-Mar-17
3	Blood	+	-/0.393	10-Mar-17	13-Mar-17
4	Blood	+	-/0.412	17-Mar-17	20-Mar-17
5	Epidural abscess*	ND	-/0.341	ND	22-Mar-17
6	Sputum*	ND	-/0.320	ND	3-Apr-17
7	Sputum*	ND	-/0.251	ND	3-Apr-17

- Negative for the IMB-ELISA
+ Positive for the test (positive *B. pseudomallei* for hemoculture and positive IMB ELISA test
ND: not determined by hemoculture
* Blood from patients with other specimen suspected to have local melioidosis were used as negative control

Table 1. (Continued)

Sample number	Specimen sites*	Method for detections		Detection date	
		Hemoculture	IMB-ELISA/OD value	Hemoculture	IMB-ELISA
8	Blood	No growth	-/0.311	15-May-17	15-May-17
9	Blood	+	+/0.801	31-May-17	31-May-17
10	Blood	No growth	-/0.315	21-Jun-17	21-Jun-17
11	Pus from wound*	ND	-/0.382	ND	28-Jun-17
12	Blood	+	+/0.790	4-Jul-17	4-Jul-17
13	Blood	+	-/0.434	16-Aug-17	16-Aug-17
14	Blood	No growth	-/0.295	3-May-17	16-May-17
15	Blood	No growth	-/0.377	7-Apr-17	17-Apr-17
16	Blood	No growth	-/0.311	9-May-17	18-May-17
17	Blood	No growth	-/0.380	7-Aug-17	9-Aug-17
18	Blood	No growth	-/0.295	14-Aug-17	18-Aug-17
19	Blood	+	+/0.550	10-June-17	12-June-17
20	Blood	+	-/0.424	13-June-17	13-June-17

- Negative for the IMB-ELISA
+ Positive for the test (positive *B. pseudomallei* for hemoculture and positive IMB ELISA test
ND: not determined by hemoculture
* Blood from patients with other specimen suspected to have local melioidosis were used as negative control

Comparison of the detection by hemoculture against IMB-ELISA

As the developed method could not reach a high sensitivity, clinical blood samples, therefore, would have to be cultured to enrich the bacteria before detection. Comparisons of the time required for detection by hemoculture and IMB-ELISA were then performed (**Table 2**). The results found that the positive culture could be detected at 15 h when the hemocultures were spiked at 10^1 and 10^2 CFU/ml, while at 10^3 CFU/ml, it could be found at the beginning (0 h). When the positive colonies were used to identify biochemical tests, another 36 h was required before the final results were obtained, whereas the IMB-ELISA method needed only 6 h. The summary of times used for positive identification results are shown in **Table 2**. The results demonstrated that although the IMB-ELISA method gave low sensitivity by itself, when used with the culture method, it gave faster results than using culture with biochemical tests.

Table 2. Comparison of the time required for positive detections by hemoculture and IMB-ELISA

Concentration of initial bacteria in spiked blood culture (CFU/ml)	Time (hrs) used for positive detections by	
	Hemoculture (with biochemical identifications)	IMB-ELISA
0	NG*	NG*
10^1	51	21
10^2	51	21
10^3	36	11

*No growth (negative control)

Discussion

Culture is still the gold standard for detection, especially when patients are suspected of having BSIs with *B. pseudomallei*. It is time-consuming (3 to 7 days) and most often the results are obtained after the patients have died.[1] Therefore, many alternative methods have been developed for rapid detection, including antibody, antigen and molecular based detection. The antibody method has problems because of a high antibody background in the endemic area.[21,22] The antigen detection gives more advantages but still has low sensitivity when used for detection in direct blood samples because of the low number of bacterial cells in the blood. LFI could be used for detection in serum, urine, and sputum but not in the blood because of the low CPS shedding and bacterial loads in the blood sample.[8] Nevertheless, after LFI was optimized, it was evaluated by detection of *B. pseudomallei* in EDTA blood samples of acutely septic patients with a sensitivity of 40%,[23] with accuracy for detection of *B. pseudomallei* in hemoculture broth (BacT/Alert, bioMérieux, Marcy L'Etoile, France).[24]

The examples for using IMB separation for improving the problem of detection are *Francisella tularensis* and *Yersinia pestis*, which are slow growth bacteria and difficult to diagnose.[10] IMB-ELISA was applied for detection of several bacteria, such as *Salmonella* in milk with a LOD of 10^5–10^6 CFU/mL,[13] *S. aureus* with a LOD of 1 ng (10^5 cells per ml),[25] and *Alicyclobacillus* spp. in apple juice with a LOD of 10^3 CFU/mL.[14] The present IMB-ELISA assay gave a LOD of the bacteria in the buffer of

10^2 CFU/ml, which is much better than other IMB-ELISAs reported and it is the first report for detection of *B. pseudomallei* in blood samples. The ELISA was combined with IMB, because it is an easy method that is suitable for use in hospitals in the endemic area. The F200 was used because it showed a lower background and a higher signal for detection than other beads (C500, S500 and S750). Moreover, the F200 beads had the maximum surface area (2.77×10^{16} nm^2) followed by S500 (1.18×10^{16} nm^2) and S750 (7.4×10^{15} nm^2). As these findings correlated with other publications that showed the larger beads have low capture and separation efficiencies, while smaller nanobeads were more effective for binding with bacterial surfaces in food samples.[26] The small beads contain faster reaction kinetics, lower mass, higher surface to volume ratios and multiple sites for binding of the target cells to the surface.[27]

While specificity was a concern (**Figure 3A**), 4B11-IMB showed specificity with *B. pseudomallei*, although there were minor cross-reactions with *E. coli*. This MAb had been reported to bind only with *B. pseudomallei* and *B. mallei*. The 4B11-MAb IgG2b subclass against the 200-kDa EPS of *B. pseudomallei* is widely used in agglutination for identification of *B. pseudomallei*.[7] This 4B11-MAb could also be used to differentiate between *B. pseudomallei* and *B. thailandensis*, which are closely related bacteria.

The reason why cross-reactions occurred with other bacteria tested is unknown. It was hypothesized that this might be due to the non-specific binding of the cross-reactive bacteria to the beads. The absorbance of *B. mallei* was nearly similar to *B. pseudomallei*, as these 2 strains are related with a 99% similarity of their genomes (9, 10). *B. mallei*, however, is not zoonotic and has never been reported in humans.[28] The other possible non-specific background might be due to the interaction between immobilized 4B11-MAb and polyclonal rabbits and/or anti-rabbits. However, we evaluated such a possibility by ELISA. The 4B11-MAb was coated onto the 96 well plates followed by polyclonal rabbits and/or anti-rabbits. The results found no reaction between them ($OD < 0.1$).

The sensitivity of optimized IMB-ELISA (**Figure 3B**) was 10^2 CFU/ml. The CE of the beads, however, was found to be nearly 100% (**Figure 2B**) when 10^2 CFU/ml bacteria were available. Therefore, the lower sensitivity might be due to the ELISA system and not the capture ability of the coated beads. Another possible factor leading to low sensitivity is the 4B11 reactive epitope, the EPS antigen. It could be shedding from the cells into the media or clinical samples.[16] The antigen concentrations might be varied in different specimens. When the time required for getting the results was compared between hemoculture and IMB-ELISA (**Table 2**), the results found that the sensitivity of the culture was higher than IMB-ELISA. Additionally, the times for identification by the biochemical tests were long, whereas the IMB-ELISA took only 6 h after sufficient growth of the bacteria. Only three positives of the IMB-ELISA were correlated with positive hemocultures. The possible reasons for low sensitivity were: 1) only 1 ml of samples were used for detection and 2) the samples were not from the same time as the hemoculture (they were EDTA blood samples taken 1–2 days after). Moreover, further development of the IMB system combined with other methods, such as Quantum dot, may increase sensitivity.[12] Quantum dots (QDs) are the new fluorescent markers or class of fluorophores or autofluorescence, which increase fluorescence intensities, long-term photostability and can be excited by a wide spectrum of wavelengths.[29] IMB-QD was used for detection of various bacteria, such as *Brucella* spp. with a LOD of 10^3 CFU/mL in 10^5 minutes.[30] In our preliminary study, IMB-QDs were used for detection of *B. pseudomallei* in positive blood samples and found that the sensitivity of inoculated blood samples was 10^4 CFU/ml when observed by the naked eye and was 10^3 CFU/ml when detected with spectrofluorometry.

In conclusion, the IMB-ELISA was successfully developed for detecting *B. pseudomallei* in blood samples. Moreover, the 4B11-IMB developed could also be used to concentrate the bacteria in the clinical specimens. Although there are still some shortcomings of the kit, such as the lower sensitivity for detection of the low numbers of bacteria in the infected blood samples, it needed 6 h before getting the result, which is not a good test for rapid diagnosis. However, its advantage might be used for concentrating the bacteria and adapted for other samples such as sputum, urine or drinking water.

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Conflict of interest

None

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