SURFACE PLASMON RESONANCE IMMUNOSENSOR FOR RAPID AND SPECIFIC DIAGNOSIS OF MELIOIDOSIS ANTIBODY

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Abstract. Melioidosis, caused by *Burkholderia pseudomallei*, is a potentially fatal disease, which requires an accurate and rapid diagnosis. This paper reports on the highly sensitive and specific detection of melioidosis antibodies by surface plasmon resonance immunosensor. The sensing surface was immobilized with *B. pseudomallei* BipD protein via a 11-mercaptoundecanoic acid self-assembled monolayer. Under optimum conditions individual sera of melioidosis patients, non-melioidosis patients (negative) and blood donors (control) were analyzed at a dilution of 1:6,000 in 10 mM phosphate buffered saline pH 7.50. The cut-off value determined from the mean ± 2SD of 20 control and 20 negative sera was 3.3 m°. At this cut-off both sensitivity and specificity were 100%. The system required only a short analysis (20 minutes) and regeneration time (12 minutes). In addition, one immobilization of the sensing surface could be reused more than 30 times. The advantages of the proposed method are savings in both time and cost of analysis, while at the same time providing excellent sensitivity and specificity.

Keywords: *Burkholderia pseudomallei*, biosensor, BipD protein

INTRODUCTION

Melioidosis is an emerging infectious disease endemic in Southeast Asia and northern part of Australia; although some sporadic cases have been reported from other subtropical and tropical areas throughout the world (Sirisinha *et al*, 2000; Tomaso *et al*, 2005). The causative bacterial agent is *Burkholderia pseudomallei*, which has high versatility to survive well in diverse environments (Cheng and Currie, 2005). In Thailand, the highest incidence of reported cases of melioidosis has been from the northeastern part of the country, with some provinces having high numbers of virulent (arabinose negative) strains obtained from soil samples (Cheng and Currie, 2005; Maharjan *et al*, 2005). In 1994 and 1995 the isolation rate of *B. pseudomallei* in northeastern hospitals was 4.2 and 4.1 per 1,000 clinical specimens respectively, compared to central, north and south hospital rate of 1.1,

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1.8, 1.1 and 1.1, 1.2 and 0.4, respectively (Leelarasamee *et al*, 1997). However, it is believed the numbers of melioidisis cases are underreported (Anuradha *et al*, 2003), as clinical manifestations of the disease are broad ranging and often difficult to differentiate from other infections (Yap *et al*, 1991). Currently the gold standard method for melioidosis diagnosis is isolation of *B. pseudomallei* from clinical specimens, a process which requires a long period and expertise (Haase *et al*, 1998; Sirisinha *et al*, 2000).

In recent years, a number of new methods to detect melioidosis have been developed in order to improve the ease of diagnosis, including polymerase chain reaction (PCR) (Hagen *et al*, 2002) and immunological methods (Cheng and Currie, 2005). Although PCR gives a more rapid response than bacterial culture (Sirisinha *et al*, 2000; Supaprom *et al*, 2007), conventional PCR assays for diagnosis of septicemic melioidosis have low sensitivity (Kunakorn and Markham, 1995; Dharakul *et al*, 1997) and low specificity (67%) (Haase *et al*, 1998). Quantitative PCR-based detection methods have greater ease of use than conventional PCR and 100% specificity (Tomaso *et al*, 2005; Novak *et al*, 2006), but sensitivity is relatively low (71%) (Supaprom *et al*, 2007). Immunological methods are generally based on detection of either *B. pseudomallei* antibodies or antigens. Methods to detect antibodies include indirect hemagglutination assay (IHA) (Hambie *et al*, 1977; Ashdown, 1981), enzyme-linked immunosorbent assay (ELISA) (Chen *et al*, 2003; Chantratita *et al*, 2007) and immunofluorescent assay (IFA) (Ashdown, 1981). Sensitivity and specificity of serum IHA is 46-94% and 34-93%, respectively, while that of serum ELISA is 74-93% and 82-97%, respectively (Cheng and Currie, 2005). IgG and IgM have been determined by ELISA, with sensitivity and specificity of about 88% and 82-83%, respectively (Dharakul *et al*, 1997).

One of the virulent factors of *B. pseudomallei*, the type III secretion system (TTSS), facilitates the organism to replicate and survive in nonphagocytic cells during its dormancy state (Brett and Woods, 2000; Stevens *et al*, 2002). TTSS is encoded by a cluster of genes *TTSS1*, *TTSS2* and *TTSS3*. *TTSS3* is required for virulence of *B. pseudomallei* in an animal model (Stevens *et al*, 2004; Warawa and Woods, 2005) and BipD protein, one of the protein products of *TTSS3*, is required for full virulence in the murine model of melioidosis (Stevens and Galyov, 2004; Stevens *et al*, 2004). Visutthi *et al* (2008) cloned and expressed BipD gene and used the recombinant protein for testing with pooled sera from melioidosis and other infections, and from normal subjects by immunoblotting, with sensitivity and specificity of 100% and 91.1%, respectively. However, the method is time-consuming (3-4 hours), and an alternative method that could provide the same or better performance with less time would be desirable. An affinity biosensor might be able to fulfill such requirements.

A biosensor consists of two components, a biological recognition element and a transducer. This combination enables measurement of the target analyte through an affinity reaction through changes in such physical properties as electrochemical (Thavarungkul *et al*, 2007), mass (Zhang *et al*, 2008) or optical (Suwansa-ard *et al*, 2009). Surface plasmon resonance (SPR), an optical detection system, occurs when a thin film of noble metal (gold or silver) is placed inside the laser beam, and when the incoming light is monochromatic and p-polarized, the

electrons of the metal oscillate and absorb energy at a certain angle of incident light, as SPR angle (Kooyman *et al*, 1988; Heideman *et al*, 1991). Binding of a target analyte to its cognate immobilized binding partner on the sensing surface causes a change in refractive index, which is proportional to the quantity of the bound analyte. This change shifts the angle where the SPR phenomenon occurs these shifts are monitored over time by the SPR detector and are recorded as a sensorgram (Yang *et al*, 2005). SPR biosensors have become a particularly powerful tool because of their high sensitivity and real-time monitoring capability (Castillo *et al*, 2004).

As BipB protein is highly specific to antibodies in melioidosis clinical serum samples (Visutthi *et al*, 2008), a biosensor using BipD to detect melioidosis antibody is highly feasible. In this work, we investigated the application of an SPR immunosensor for the detection of the melioidosis antibodies in serum by immobilizing BipD on a sensing surface and measuring the SPR angle shift caused by the antibody binding.

MATERIALS AND METHODS

Materials

BipD gene (GenBank, accession no. EF120623; provided by the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University) was cloned and expressed as previously described (Visutthi *et al*, 2008). 11-Mercaptoundecanoic acid was obtained from Sigma-Aldrich (St Louis, MO), bovine serum albumin from Fluka (Buchs, Switzerland), and all other chemicals were of analytical grade. Buffers were prepared with deionized water and were filtered through a Millipore filter, pore size 0.22 µm with subsequent degassing. Serum samples were obtained from Songklanagarind Hospital, Hat Yai, Songkhla, Thailand. Positive serum samples were from bacteremia inpatients with a positive culture of *B. pseudomellei*. Negative sera were from patients with bacteremia caused by other bacterial species (non-melioidosis). Control sera were from blood donors who had tested negative for AIDS, hepatitis B and syphilis. Pooled positive serum was a mixture of 10 serum samples from melioidosis patients while pooled negative serum was from 10 non-melioidosis patients. These pooled sera were used to optimize the SPR immunosensor system. Individual serum samples, 20 positives, 20 negatives and 20 controls were used to test the performance of the analysis system.

Immobilization of BipD

A gold disk was exposed to a freshly prepared piranha solution [3:1 (v/v) mixture of H_2SO_4 and H_2O_2] for 3 minutes and then rinsed with ethanol and distilled water. The disk was dried with pure nitrogen gas before being inserted into the cuvette block of the SPR device (AutoLab Spirit®, Metrohm Autolab B.V., The Netherlands). An 11-mercaptoundecanoic solution (150 mM in absolute ethanol) was injected onto the surface of the gold disk and incubated for 5 hours, after which it was rinsed with ethanol and distilled water, respectively. The disk surface then was activated with a solution of 0.2 M *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) in distilled water for 50 minutes and rinsed with carrier buffer (see below). A 100 µl aliquot of 0.10 mg ml^{-1} BipD in 10 mM acetate buffer pH 5.0 was placed on the activated surface and left for the reaction to take place for 2 hours. Finally, the electrode was treated in ethanolamine for 7 minutes and 1%

bovine serum albumin (BSA) solution for 20 minutes to block the surface from any non-specific binding.

SPR measurement

After the immobilization of BipD a flow system was applied for the analysis (Fig 1). A micro syringe pump (KD Scientific, Holliston, MA) was used to deliver the carrier buffer over the sensing surface inside the cuvette block at a flow rate of 15 µl min^{-1} (Suwansa-ard *et al*, 2009). The SPR angle was monitored by an Autolab SPRINGLE SPR (AutoLab Spirit®, Metrohm Autolab B.V., The Netherlands) connected to a computer. When a sample was injected (300 µl) the binding between the immobilized BipD and melioidosis antibody could be observed by the shift of the SPR angle.

Regeneration process

In order to investigate the optimal regeneration solution and carrier buffer, a screening of the dilution factor was first studied. This was to minimize matrix interference. Pooled negative and positive sera were diluted 1,000, 2,000, 5,000, 6,000, 8,000, 10,000, 100,000, 500,000 and 1,000,000 times in 15 mM phosphate buffer pH 7.20. The diluted samples were injected into the SPR system to determine a dilution factor that produced with the pooled negative serum the same response as a blank sample (injected buffer), together with a clear separation between pooled negative and positive signals.

The optimal condition for each parameter (pH and type of regeneration solution and concentration and pH of a running buffer) (Table 1) was a balance between SPR angle shift and analysis time. Optimizations were carried out by varying one parameter at a time, while the others were kept constant. In these studies diluted pooled negative and positive sera were employed with three replicates performed for each tested value. The starting operating conditions of the SPR biosensor system were a flow rate of analyte of 15 µl min^{-1}, a flow rate of regeneration solution of 175 µl min^{-1} and a 300 µl sample volume (Suwansa-ard *et al*, 2009). When the optimum value of one parameter was obtained, it was used to optimize the next parameter.

Dilution

A large dilution will ensure a minimal matrix effect and low background signal; however, too much dilution can also

Table 1
Optimum conditions of regeneration solution and running buffer in flow system of SPR immunosensor.

Parameter	Test	Optimum
Regeneration solution		
Type	50 mM NaOH HCl pH 2.50 (3.16 mM) 50 mM glycine-HCl pH 2.50	50 mM NaOH
Concentration (mM)	10, 25, 50, 75	25 mM NaOH
Carrier buffer		
Concentration of PBS[a] (mM)	5, 10, 15, 25, 50, 100	10 mM PBS
pH of PBS[a]	6.50, 7.00, 7.20, 7.40, 7.50, 7.60, 7.80, 8.00	7.50

[a]Phosphate buffered saline ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ containing 150 mM NaCl)

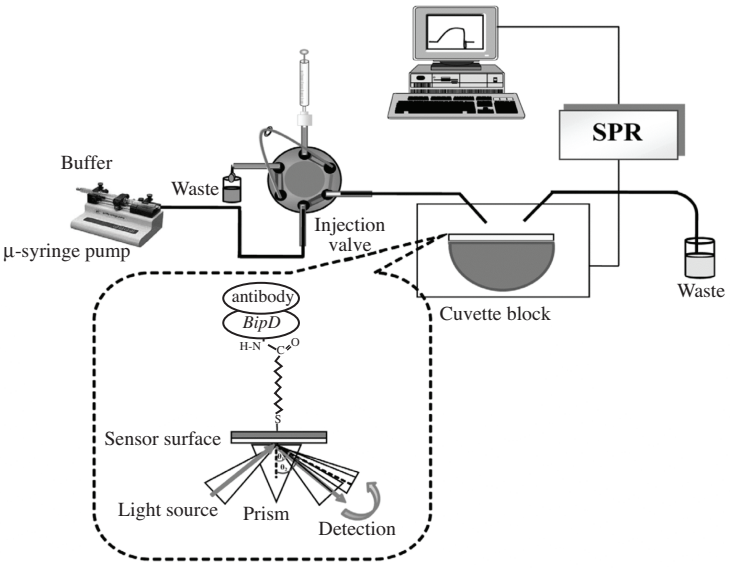

Fig 1–Schematic diagram showing the flow SPR immunosensor system and SPR angle measurement at the sensing surface.

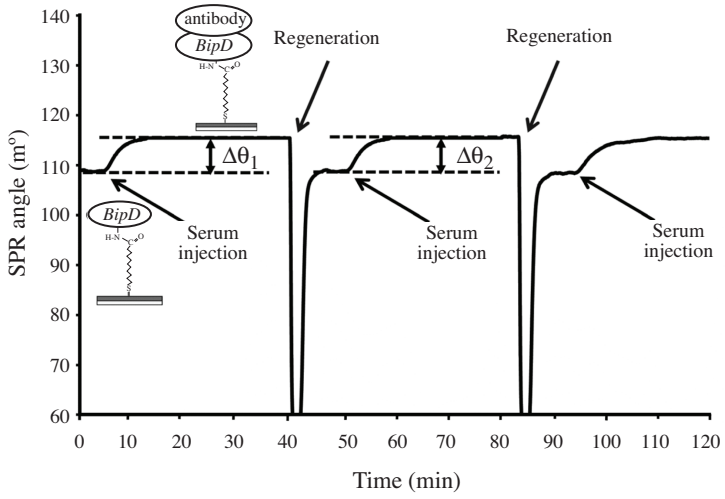

Fig 2–SPR sensorgram. This was obtained from injection of diluted pooled positive serum over immobilized BipD resulting in the increase of SPR angle ($\Delta\theta_1$). After the regeneration solution was injected to disrupt binding between melioidosis antibody and BipD the next analysis was performed to obtain a new signal ($\Delta\theta_2$).

reduce signal of the analyte. Therefore, dilution factors between 2,500 and 20,000 were evaluated, using the optimal conditions obtained as described in the previous section.

Melioidosis detection

Melioidosis in individual serum samples was analyzed by bacterial culture (conducted at Songklanagarind Hospital, Hat Yai, Songkhla, Thailand) and compared with the SPR biosensor system. In the case of the SPR biosensor analysis, these samples were diluted and measured under the optimal conditions as described above.

Statistical analysis

Results were validated using the Mann-Whitney rank-sum test. Significant difference between two samples is accepted when $p < 0.05$.

RESULTS

Regeneration solution and carrier buffer

Carrier buffer was first passed through the system until a baseline was obtained. When a sample was injected, antibodies in the sample bound to the immobilized BipD causing the SPR angle to increase (Fig 2). Antibodies on the surface then were removed using the regeneration solution and the

surface was used for a new analysis. Initial screenings of the dilutions showed that at 6,000-fold dilution the SPR angle shift from the pooled negative sample (1.28 ± 0.08 m°) was the same as the blank sample (1.25 ± 0.05 m°), and was much lower than the pooled positive serum (8.40 ± 0.97 m°). Therefore, the pooled positive sample was diluted by a factor of 6,000 using 15 mM phosphate buffered saline (PBS) pH 7.20 ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ containing 150 mM NaCl) for the following studies.

The regeneration solution was used to break the binding between the immobilized BipD and melioidosis antibodies in the serum so that the sensing surface could be reused. The evaluation of the performance of the regeneration solution was conducted by considering the residual activity of the immobilized BipD, calculated from the SPR angle shift resulting from the binding between melioidosis antibodies and BipD before ($\Delta\theta_1$) and after ($\Delta\theta_2$) regeneration (Fig 2) as follows:

$$\text{Residual activity (\%)} = \frac{\Delta\theta_2 \times 100}{\Delta\theta_1}$$

The type of regeneration solution was first studied using 50 mM glycine-HCl pH 2.50, HCl pH 2.50 (3.16 mM) and 50 mM NaOH. Residual activity was 81 ± 8% for glycine-HCl, 73 ± 9% for HCl and 91 ± 5% for NaOH solutions. The regeneration time was 10 minutes for HCl and 12 minutes for glycine-HCl and NaOH solutions. NaOH was chosen for further study since it gave the highest residual activity.

NaOH concentration of 10, 25, 50 and 75 mM was then studied resulting, in percent residual activity of 85 ± 5, 95 ± 4, 91 ± 4 and 81 ± 5, respectively. Therefore, 25 mM NaOH was chosen as the regeneration solution as it gave the highest percent residual activity.

PBS, which is widely used as a carrier buffer for SPR immunosensor systems (Bravman *et al*, 2006) was employed in this study. Concentrations of PBS pH 7.20 were investigated at 5, 10, 15, 25, 50 and 100 mM. The SPR angle shift increased with increase in concentrations from 5 to 10 mM_2 and then decreased. Ten mM was used to study carrier buffer pH at 6.50, 7.00, 7.20, 7.40, 7.50, 7.60, 7.80 and 8.00. The SPR angle shift increased with increase in pH until it reached the highest response at pH 7.50, after which the signal decreased. Thus, 10 mM PBS pH 7.50 was used in the following experiments.

Dilution effect

Under the optimal regeneration solution and carrier buffer conditions, the responses of the system to the pooled negative and positive sera were studied again, with dilutions between 2,500 to 20,000 times. When the dilution was lower than 6,000 times the pooled negative and positive samples gave similar signals (Fig 3). This was most likely due to matrix interference. At dilutions of 6,000 and higher, response of the pooled negative serum was well separated from that of the pooled positive serum. Thus, a dilution of 1:6,000, which provided the highest pooled positive response, was chosen for further analysis of individual serum samples.

Stability

Under the optimal conditions established (Table 1), stability of the BipD was studied by repeated injections of diluted pooled positive serum with subsequent injections of 25 mM NaOH, 20 times per day for 2 days and percent residual activity of each injection was calculated. During

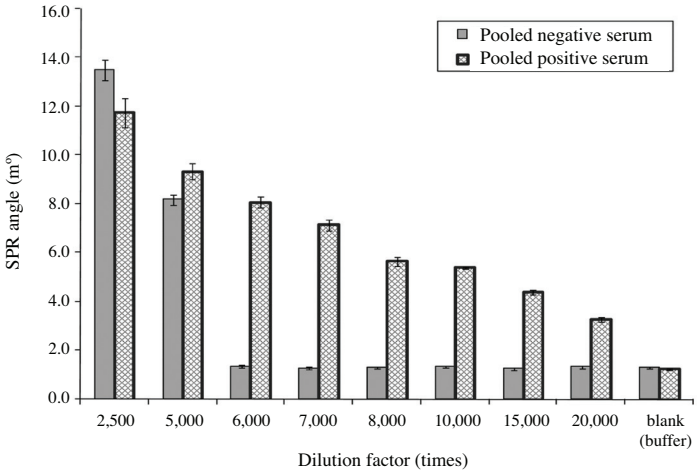

Fig 3–Effect of dilution on response of pooled negative and positive sera. Experiments were conducted as described in legend to Fig 2.

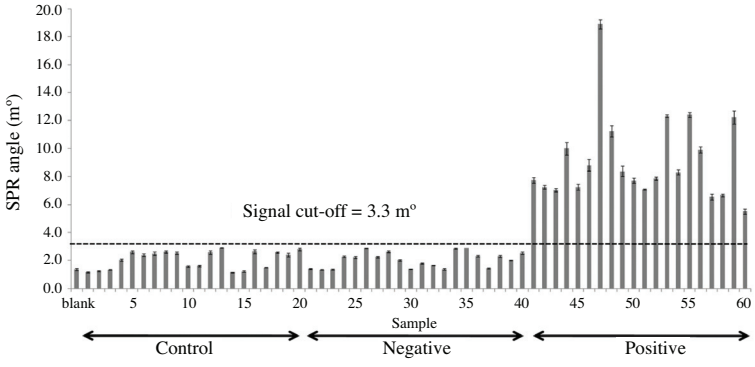

Fig 4–SPR angle shift from individual sera. Experiments were conducted as described in legend to Fig 2.

the first 32 regenerations the percent residual activity was 97 ± 5 (5.1 % RSD) after which the response decreased rapidly. The decreased response could have been the result of the loss of BipD reactivity or the loss of the self-assembled monolayer. However, baseline of the response signal was similar to the one before the modified electrode was first used, indicating that the self-assembled monolayer was still intact. Thus, the decrease of residual activity was most likely due to the loss of BipD reactivity.

Determination of melioidosis in individual samples

Individual serum samples were analyzed under the optimum conditions. The average response of both control and negative serum samples was 2.1 ± 0.6 m° (Fig 4). The average response of the positive samples was 9.2 ± 3.1 m°. The signal cut-off between negative and positive serum samples was determined as mean + 2SD [2.1 + 2 (0.6) m°], giving a value of 3.3 m°. Using this cut-off value sensitivity and specificity of this technique were both 100%.

Comparison of results

The results of the control, negative and positive individual sample tests were compared using the Mann-Whitney rank-sum test. Comparison between control and negative individual sample results showed no difference. However, when the negative and positive individual sample results were compared, a significant difference ($p < 0.05$) was found, indicating that positive individual samples could be clearly separated from the negative and the control individual serum samples by the SPR immunosensor test.

DISCUSSION

Melioidosis remains a serious public health problem in endemic areas. Current research is focused on developing a rapid test that can speed up the diagnosis time, as the conventional bacterial culture leads to delayed diagnosis resulting in serious outcome. The work described in this report is one such attempt. Flow SPR immunosensor system with immobilized BipD protein developed could detect melioidosis antibodies in serum with 100% sensitivity and specificity. In addition, only a very small amount of serum is required since the analysis can be carried out with a dilution of 1:6,000. Furthermore, this technique provided a response in only 20 minutes, allowing proper treatment to be started much sooner for infected patients. Another advantage is that one electrode preparation can be used up to 30 times, helping to save time and reduce the cost of analysis, with a cost per sample (three injections) of about USD 0.55.

Although the number of samples tested in this study was relatively small, the results indicated that the method shows high potential. From 20 negative and 20 control samples the cut-off value (3.3 m^o) was much lower than the average response from the positive samples (9.2 ± 3.1 m^o). Therefore, this technique would be applicable when samples from a larger population are tested. The flow SPR immunosensor system is undergoing modification for use in the field. However, not all melioidosis-infected patients may develop sufficient level of antibodies to BipD antigen that could be detected by method. SPR immunosensor immobilized with purified antibodies to *B. psueudemallei* surface antigens would be another approach for detection of melioidosis cases.

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