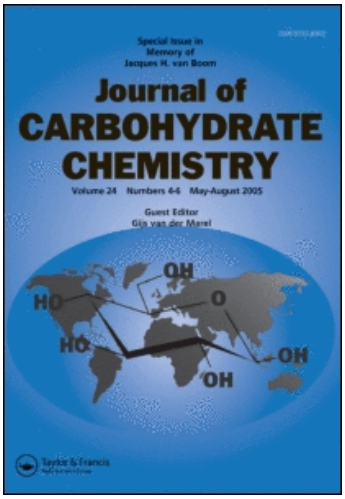

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Application of Polysaccharide Microarray Technology for the Serodiagnosis of *Burkholderia pseudomallei* Infection (Melioidosis) in Humans

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Burkholderia pseudomallei is the causative agent of melioidosis, a bacterial infection endemic in tropical regions including southeast Asia and northern Australia. *B. pseudomallei* contains structurally unique polysaccharides (capsular polysaccharide and *O* – antigen saccharides of lipopolysaccharide). A polysaccharide microarray platform was developed by immobilizing these polysaccharides onto glass slides. Employing this microarray, we were able to demonstrate the presence of antibodies to these polysaccharide antigens in the sera of melioidosis patients, but not in serum from nonmelioidosis human subjects. The advantages of this polysaccharide microarray technology over the conventional tests for the serodiagnosis of melioidosis are discussed.

Keywords Polysaccharide microarray, *Burkholderia pseudomallei*, Serodiagnosis, Melioidosis

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INTRODUCTION

Melioidosis is a severe infection caused by *Burkholderia pseudomallei*, a motile gram-negative bacillus. *B. pseudomallei* is generally found in water or moist soil in tropical regions, including southeast Asia and northern Australia, where melioidosis is endemic.[1] The severity of melioidosis ranges from a septicemic illness with rapid death to a chronic illness characterized by fever and weight loss. Isolation of *B. pseudomallei* from patient samples is the "gold standard" for diagnosis of melioidosis, but this requires selective media and can take up to 2 days.[2] In addition to culture-based techniques, the indirect hemagglutination assay (IHA), latex agglutination, and immunofluorescence are the most common methods used worldwide for the diagnosis of melioidosis.[2] IHA involves the use of a crude bacterial extract as the antigen source; therefore, the potential for false diagnosis cannot be ruled out. A serodiagnostic assay based on pure antigens is a primary requisite for correct serodiagnosis of human melioidosis. Recently,[3] we developed a *Burkholderia* polysaccharide microarray platform by immobilizing pure polysaccharide antigens derived from *B. mallei* and *B. pseudomallei*. This polysaccharide microarray was successfully used to detect anticapsule antibodies in the serum of a rabbit vaccinated with purified *B. pseudomallei* capsular polysaccharide and in the convalescent serum from a human infected with *B. mallei*.[4] In this study, we extended the application of *Burkholderia* polysaccharide microarray technology for the serodiagnosis of melioidosis in human patients.

EXPERIMENTAL

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *B. pseudomallei* 1026b, *B. pseudomallei* 576, and *B. pseudomallei* SRM117. Strain 1026b produces a lipopolysaccharide (LPS) *O*-antigen with a typical banding pattern (type A), whereas strain 576 produces an atypical banding pattern (type B).[5,6] SRM117 is a derivative of 1026b that does not produce *O*-antigen saccharides.[7] Bacterial strains were cultured under the growth conditions described previously.[3]

Human Sera

Serum samples were obtained on admission from adult patients presenting with suspected melioidosis to Sappasithiprasong Hospital, Ubon Ratchathani, Northeast Thailand, between June and October 2004. Patients with suspected melioidosis were identified by active ward surveillance and multiple samples were taken for culture. Isolation of *B. pseudomallei* from any sample was

considered diagnostic. Ethical approval was obtained from the Ministry of Public Health, Royal Government of Thailand.

Polysaccharide Microarray

A Burkholderia polysaccharide array platform was prepared by a procedure described previously.[3] Briefly, polysaccharides (capsular polysaccharide and LPS *O*-antigen saccharides) were isolated from the bacteria by hot-phenol extraction, DNAse, RNase, and proteinase K digestion, followed by acetic acid hydrolysis. They were converted to glycosylamines in the presence of ammonium acetate by reductive amination. The neutral sugar contents of the polysaccharides were estimated by phenol-sulfuric acid method[8] using L-rhamnose as the standard. The glycosylamines were printed on glass slides (Super Epoxy in 16 wells, NUNC Inc.) using a robotic microarrayer (VIRTEK, Chip Writer Pro). Custom printing was carried out by Kam Tek, Inc. (Gaithersburg, MD). The polysaccharide antigens (1 μmole/mL rhamnose equivalents) were reconstituted in TBS (Tris-buffered saline, 25 mM Tris, 0.15 M NaCl, pH 7.2) and arrayed as six replicates. The procedure, including blocking the slides, washing, and incubating them with serum, was essentially the same as that described previously.[3] Human serum was used at a serial dilution starting from 1:1000 (v/v) to 1:64,000 (v/v). Each dilution was used in duplicate, resulting in replicates of 12 for each polysaccharide antigen. Antihuman IgG (H + L) (KPL, Gaithersburg, MD) secondary antibody was labeled with Cy3, a bifunctional NHS-ester dye using the Amersham Biosciences kit. The secondary antibody was used at a 1:1000 (v/v) dilution in TBS. After washing, the slides were scanned for fluorescence at 532 nm with a Gene Pix 4000B Axon Scanner, using the software Gene Pix Pro 5.1. The fluorescence intensity of each spot was also measured. We also used inulin (Sigma) as a negative control (1 μmole/mL glucose equivalents).

RESULTS AND DISCUSSION

Carbohydrate microarray, or glycan microarray or polysaccharide microarray, is an emerging area of research.[9–12] This type of microarray was previously employed to study protein and carbohydrate interactions and to diagnose bacterial and viral infections.[13,14] Recently, Wang et al.[15] applied photogenerated glycan arrays to identify immunogenic sugar moieties of the exosporium, the outer layer of *Bacillus anthracis*, the causative agent of anthrax. Most *B. pseudomallei* strains produce a capsular polysaccharide and a type A or type B LPS *O*-antigen.[6,16,17] The type A *O*-antigen polysaccharide is an unbranched high-molecular-weight polymer of repeating units of 3)-β-D-glucopyranose-(1, 3)-6-deoxy-L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents. The chemical structure of the

type B LPS *O*-antigen is currently unknown, but it is serologically distinct from the type A LPS *O*-antigen and is present in only about 2% of *B. pseudomallei* strains.[6] The capsular polysaccharide is a homopolymer of 2-*O*-acetyl-6-deoxy-β-D-*manno*heptopyranose. *B. pseudomallei* 1026b produces capsular polysaccharide and a type A LPS *O*-antigen saccharide. *B. pseudomallei* 576 also produces the capsular polysaccharide and an atypical LPS *O*-antigen (type B).[18] *B. pseudomallei* strain SRM117 lacks *O*-antigen saccharide;[7] however, it produces the capsular polysaccharide. Previously,[3] we prepared a polysaccharide microarray platform using these antigens derived from *B. pseudomallei*. In the present study, we used the same platform for detecting saccharide antibodies in human melioidosis sera. Figure 1 shows a typical *B. pseudomallei* polysaccharide microarray probed with 14 human melioidosis serum samples and nine nonmelioidosis serum samples. All the melioidosis serum samples strongly immunoreacted with the purified 1026b, 576, and SRM117 polysaccharides as compared to nonmelioidosis serum samples (Fig. 1). It is evident that the melioidosis sera contained antibodies directed against capsular polysaccharide because there was strong reactivity with

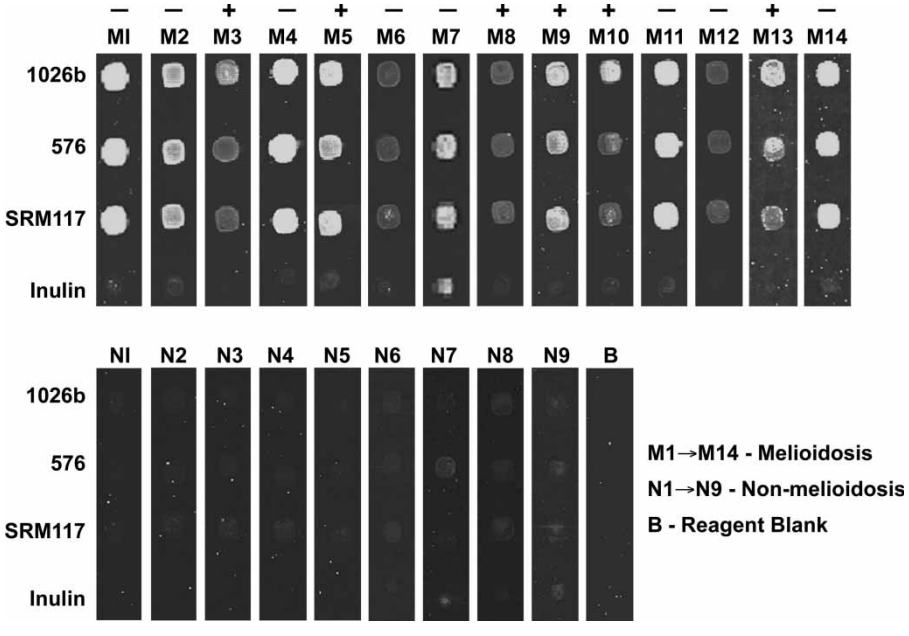

Figure 1: Typical immunoreactivity of *Burkholderia* polysaccharide microarray with human sera. Isolated polysaccharides from the bacteria were printed in replicates of 12 at a concentration of 1 μmole/mL rhamnose equivalents. The array was probed with different human sera at 1:4000 (v/v) dilution. Immunoreactivity was also seen at serial dilutions from 1:1000(v/v) to 1:64,000 (v/v) as well (diagram not shown). Cy3-labeled secondary antibody was reacted with the microarrays as described in the text. Symbols – and + indicate the absence and presence of bacteria in the serum sample, respectively.

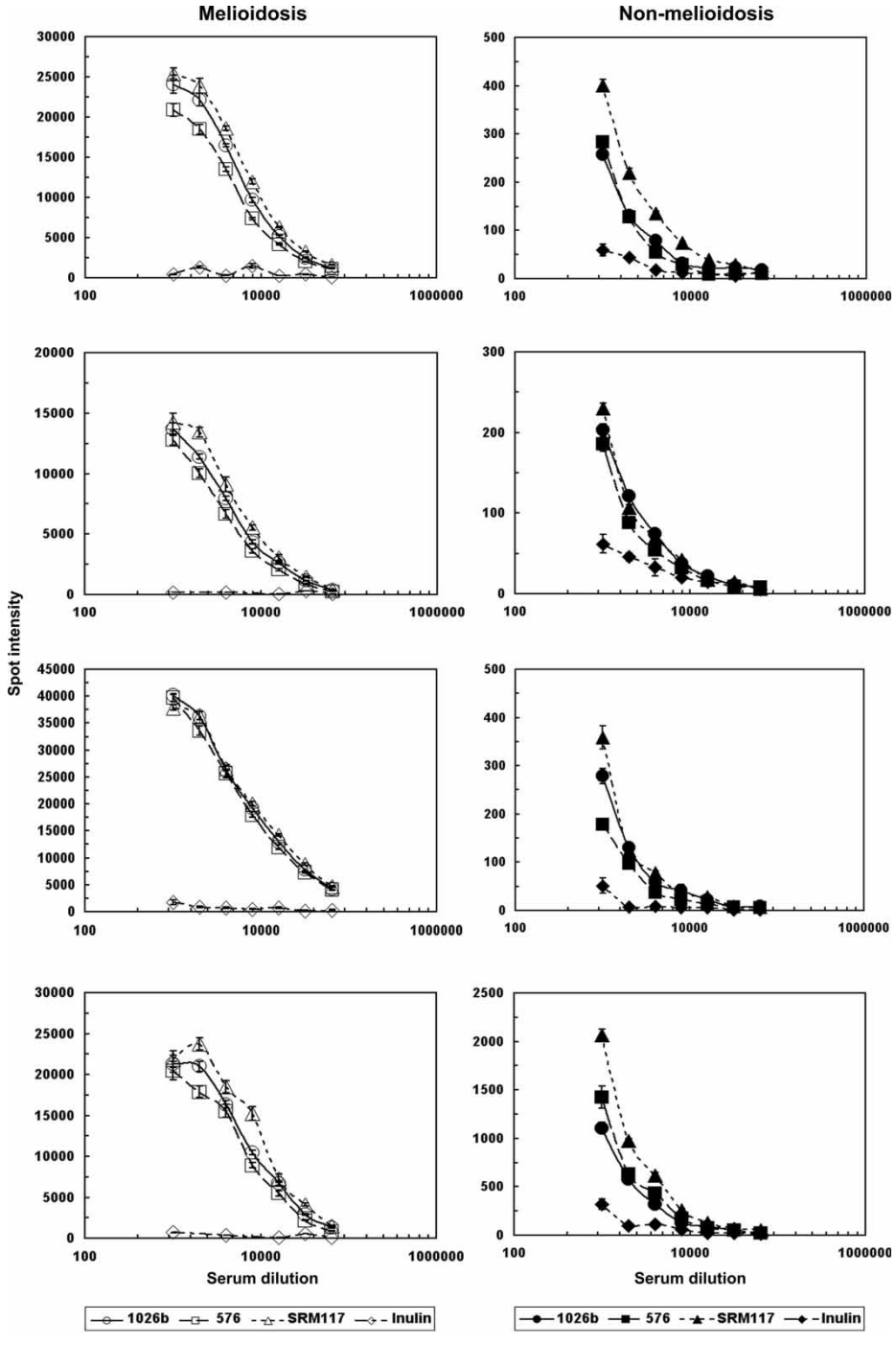

Figure 2: Typical titration curves generated by probing *Burkholderia* polysaccharide microarrays with human sera. Human sera were used at a serial dilution from 1:1000 v/v to 1:64,000 v/v. Results are expressed as means of 12 replicates ± SEM.

SRM117, a strain that does not produce an LPS *O*-antigen. It is noteworthy that the melioidosis serum samples also reacted strongly with the polysaccharides isolated from *B. pseudomallei* 576, a strain that produces an atypical LPS *O*-antigen (type B). It is likely that the reactivity in these samples was directed against the capsular polysaccharide as the majority of melioidosis patients are infected with strains containing type A LPS *O*–antigen.[6] In contrast, antibodies against both capsular polysaccharides and *O*-antigen saccharides were absent in normal or nonmelioidosis sera (Fig. 1).

Antibody titers in human melioidosis sera are conventionally determined by the IHA test.[2] The source of the antigen in the IHA test is the supernatant fluid of a heat-killed bacterial culture broth. An initial higher concentration of human serum is used in the IHA test (1:10 v/v) relative to the polysaccharide microarray technology described here. Both of these parameters could contribute to the false diagnosis of melioidosis. These problems were circumvented in the polysaccharide microarray by employing pure polysaccharide antigens as well as using an initial dilution of human serum at 1:1000 (v/v). Figure 2 shows the typical titration curves for melioidosis versus nonmelioidosis sera. Using the polysaccharide microarray, depending on the serum titer of the melioidosis patient, it was possible to detect polysaccharide antibodies even at very high dilution (1:16,000 to 1:32,000 v/v).

We next examined the immunoreactivity of melioidosis sera from various patients as compared to sera from nonmelioidosis individuals. We chose the spot intensity of the microarray of each polysaccharide antigen after probing with serum at 1:4000 v/v dilution (Fig. 3). The spot intensities of 12 replicates for each antigen from each patient were modeled on the basis of a

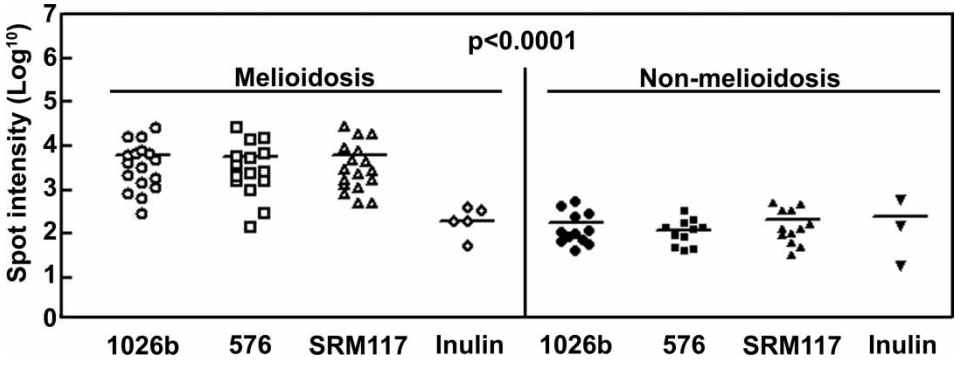

Figure 3: Comparison of the immunoreactivities between melioidosis and nonmelioidosis sera toward *Burkholderia* polysaccharide antigens. The spot intensities of the polysaccharide microarray (Fig. 1) were measured. Each data point is the geometric mean of replicates of 12 of each patient. Sera from 17 melioidosis patients and 12 nonmelioidosis subjects were indicated for each polysaccharide antigen (1026b, 576, SRM117, and inulin). Data points less than the expected number of patients (see values for inulin in the melioidosis and nonmelioidosis groups) indicate that the spot intensity was zero.

Table 1: Comparison of spot intensity[a] of polysaccharide microarrray between melioidosis and nonmelioidosis patients.

Polysaccharide	Melioidosis	N[b]	Nonmelioidosis	N[b]
1026b	3419.43 ± 1.35	17[c]	129.78 ± 1.27	12[c]
576	2782.81 ± 1.44	15	107.51 ± 1.22	11
SRM117	3162.01 ± 1.35	17	144.36 ± 1.29	12
Inulin	189.87 ± 1.44	5	121.21 ± 2.71	3

[a]Spot intensity was expressed as geometric mean ± SEM.
[b]The total number of patients in the group.
[c]Indicates the number of values acceptable for calculation. The remainder of the values did not fall into the linear portion of the regression curve including zero values.

four-parameter logistic regression curve. The data points at 1:4000 v/v dilution fell on the linear portion of the curve and therefore, spot intensities at this dilution were chosen. The geometric mean of the spot intensity for each polysaccharide antigen from the bacteria 1026b, 576, SRM117, and inulin was calculated and compared between melioidosis and nonmelioidosis sera (Table 1). A Student's t-test indicated that there was a significant difference ($p < 0.0001$) in the spot intensity between these two groups of patients for each polysaccharide antigen (1026b, 576, and SRM117).

In summary, we applied polysaccharide microarray technology for the serodiagnosis of melioidosis in human patients. This technology could circumvent certain problems in serodiagnosis of melioidosis, especially in cases where the patients may present with clinical features of melioidosis but their sera are culture negative.[1] Some of the melioidosis patients might be on antibiotic treatment and may have false-negative cultures. The polysaccharide microarray technology could play a clear role in the diagnosis of melioidosis in this group of patients. Furthermore, this technology could be an alternative or complementary technology to IHA for the serodiagnosis of melioidosis, especially in instances where the isolation of the bacteria from serum poses problems. Even though the cost of this technology is higher than the IHA test, this technology could be adopted in the laboratories routinely performing DNA microarrays, where a scanner (Gene Pix, Perkin Elmer or from any other company) is available for use.

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