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Application of carbohydrate microarray technology for the detection of *Burkholderia pseudomallei*, *Bacillus anthracis* and *Francisella tularensis* antibodies

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ABSTRACT

We developed a microarray platform by immobilizing bacterial 'signature' carbohydrates onto epoxide modified glass slides. The carbohydrate microarray platform was probed with sera from non-melioidosis and melioidosis (*Burkholderia pseudomallei*) individuals. The platform was also probed with sera from rabbits vaccinated with *Bacillus anthracis* spores and *Francisella tularensis* bacteria. By employing this microarray platform, we were able to detect and differentiate *B. pseudomallei*, *B. anthracis* and *F. tularensis* antibodies in infected patients, and infected or vaccinated animals. These antibodies were absent in the sera of naïve test subjects. The advantages of the carbohydrate microarray technology over the traditional indirect hemagglutination and microagglutination tests for the serodiagnosis of melioidosis and tularemia are discussed. Furthermore, this array is a multiplex carbohydrate microarray for the detection of all three biothreat bacterial infections including melioidosis, anthrax and tularemia with one, multivalent device. The implication is that this technology could be expanded to include a wide array of infectious and biothreat agents.

1. Introduction

There is a growing interest in carbohydrate, glycan or polysaccharide microarray technology focusing on cell surface carbohydrate–protein interactions, as well as the diagnosis of bacterial and viral infections.[1–7] Recently, we developed a *Burkholderia* polysaccharide microarray technology based on bacterial polysaccharide antigens.[8,9] By employing this technology,[8] we were able to detect anti-capsule antibodies in the serum of a rabbit vaccinated with purified capsular polysaccharide from *B. pseudomallei*, the causative agent of melioidosis. We were also able to detect capsular antibodies in the convalescent serum from a human infected with *B. mallei*, the causative agent of glanders. Furthermore, a *B. pseudomallei* polysaccharide microarray platform was employed for the serodiagnosis of melioidosis in humans.[9] In this paper, we extend the application of this carbohydrate microarray technology to the detection of antibodies against *Francisella tularensis* and *Bacillus anthracis*, the causative agents of tularemia and anthrax, respectively. In particular, the multivalent carbohydrate microarray platform developed in this study was used to specifically detect antibodies in the serum of melioidosis patients or animals immunized with bacteria that cause tularemia or anthrax.

2. Results and discussion

2.1. Chemical nature of carbohydrate antigens used

2.1.1. *B. pseudomallei*

Almost all *B. pseudomallei* strains, including 1026b and 576, produce a capsular polysaccharide that is a homopolymer of 2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose.[10–14] They also produce a type A or type B lipopolysaccharide (LPS) O-antigen.[12,15,16] The type A O-antigen polysaccharide is an unbranched high molecular weight polymer of repeating units of 3)-β-D-Glc*p*-(1→3)-6-deoxy-L-Tal*p*-(1→, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents. The structure of type B LPS O-antigen polysaccharide is currently unknown. *B. pseudomallei* strain, SRM117 (a 1026b LPS mutant), produces the capsular polysaccharide but lacks

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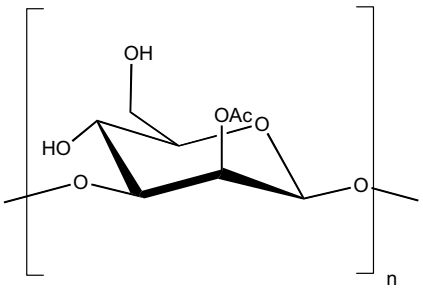

Figure 1A. Capsular polysaccharide (homopolymer of 2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose) derived from *B. pseudomallei*.

an O-antigen saccharide.[17] In summary, capsular polysaccharide (Fig. 1A) and O-antigen polysaccharide (Fig. 1B) derived from *B. pseudomallei* were used as carbohydrate antigens. These antigens were used as glycosylamine derivatives.

2.1.2. *B. anthracis*

Spores of *B. anthracis*, the causative agent of anthrax, are enclosed by a prominent loose-fitting layer called the exosporium.[18–20] A highly immunogenic (collagen-like) glycoprotein called BclA is the major component of the nap-like hairs that protrude from the exosporium. BclA contains two O-linked oligosaccharides, a 715 Da tetrasaccharide and a 324 Da disaccharide. The non-reducing terminal sugar of the tetrasaccharide was found to be 4,6-dideoxy-4-(3-hydroxy-3-methylbutamido)-2-*O*-methyl-D-Glc*p*. This sugar was termed anthrose[21] because of its exclusive presence in *B. anthracis*. This sugar was absent in *Bacillus cereus* or *Bacillus thuringiensis*. In our study, we used a synthetic anthrose[22] (Fig. 2A) and a corresponding tetrasaccharide [2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-L-Rha*p*] (Fig. 2B) attached to a linker.

2.1.3. *F. tularensis*

F. tularensis, is the causative agent of tularemia.[23] *F. tularensis* subspecies *tularensis* (type A) and holarctica (type B) are the major subspecies, with the former being highly virulent in humans.[24] A live vaccine strain (LVS) of *F. tularensis* is empirically derived from a virulent strain of type B.[24] The O-antigen of LPS derived from *F. tularensis* subsp. *holarctica* contains the rare sugars 2-acetamido-2,6-dideoxy-D-Glc*p* (QuiNAc) and 4,6-dideoxy-4-formamido-D-Glc*p* (Qui4NFm) and two moles of 2-acetamido-2-deoxy-D-galacturonamide (Gal*p*ANAc), to give the repeat structure, 4-α-D-Gal*p*ANAc-(1→4)-α-D-Gal*p*ANAcN-(1→3)-β-QuiNAc-(1→2)-β-Qui4NFm.[25] The O-antigen of *F. tularensis* subsp. *tularensis* SCHU S4 had been shown[26] to contain the repeat structure, (1→2)-D-Qui4NFm-(1→4)-D-Gal*p*ANAc-(1→4)-D-Gal*p*ANAc-(1→3)-D-QuiNAc. In the present study, a trisaccharide, α-D-Gal*p*ANAc-(1→4)-α-D-Gal*p*ANAc-(1→3)-D-QuiNAc (Fig. 3), derived from *F. tularensis* strain 15 was used. This trisaccharide was used as a glycosylamine derivative.

2.2. Carbohydrate microarray platform

The capsular and O-antigen polysaccharides from *B. pseudomallei* and the trisaccharide derived from *F. tularensis* were converted to glycosylamines by reductive amination. The glycosylamines were then immobilized on epoxide-functionalized glass slides.[9] Anthrose and the corresponding tetrasaccharides attached to a linker containing $-NH_2$ group were also printed on the glass slides.

2.3. Probing of carbohydrate microarray platform with antibacterial serum

Figure 4 shows a typical carbohydrate microarray platform probed with (a) nine human melioidosis serum samples and four non-melioidosis serum samples, (b) serum samples obtained from four anthrax-infected rabbits and four control rabbits and (c) serum samples obtained from four tularemia-infected rabbits and

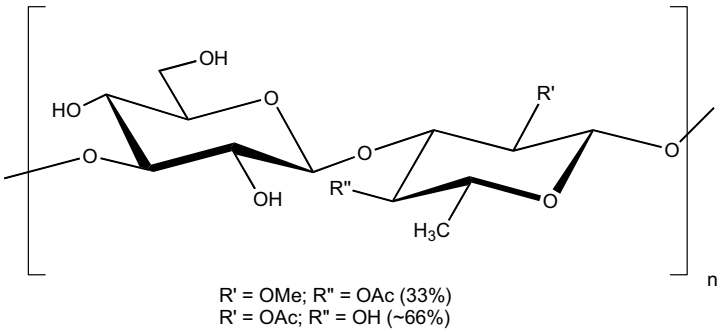

Figure 1B. O-Antigen polysaccharide [3)-β-D-Glc*p*-(1→3)-6-deoxy-L-Tal*p*-(1→]$_n$ derived from *B. pseudomallei*.

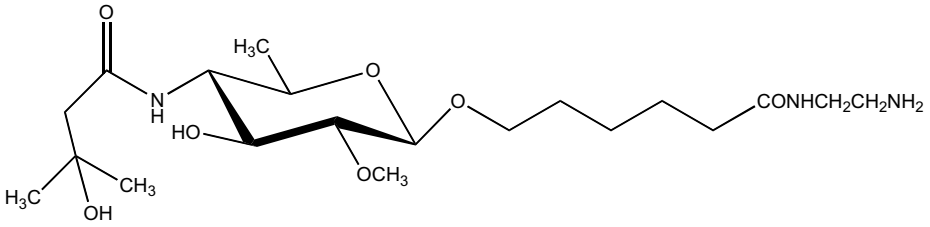

Figure 2A. Anthrose (4,6-dideoxy-4-(3-hydroxy-3-methylbutamido)-2-*O*-methyl-D-Glc*p*) attached to a linker.

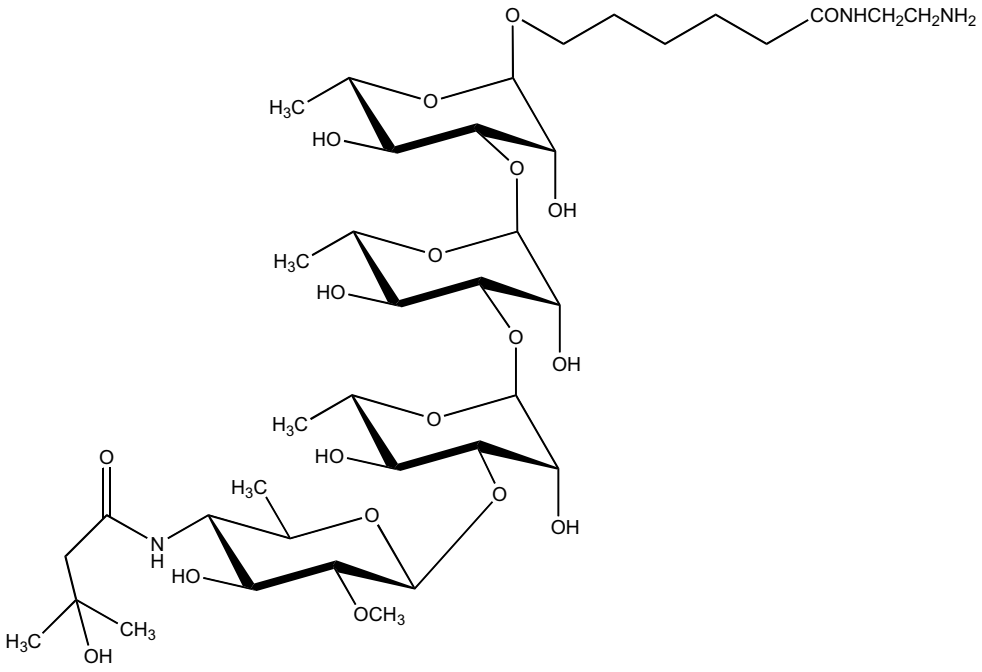

Figure 2B. Anthrose tetrasaccharide ([2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-L-Rha*p*]) attached to a linker.

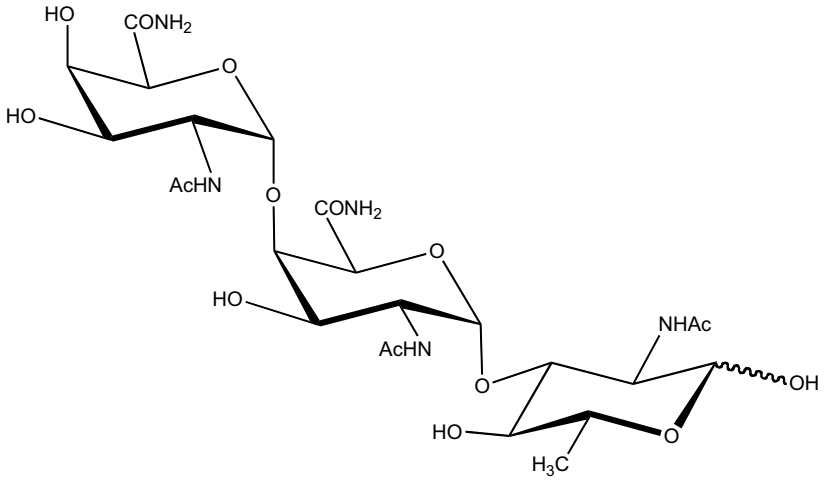

Figure 3. Trisaccharide (α-D-Gal*p*ANAc-(1→4)-α-D-Gal*p*ANAc-(1→3)-D-QuiNAc) derived from *F. tularensis.*

two control rabbits. Human melioidosis samples showed strong immunoreactivity with polysaccharides derived from *B. pseudomallei* strains 1026b, 576 and SRM117, indicating that the antibodies are directed towards capsular polysaccharide and O-antigen saccharides. In contrast, normal or non-melioidosis serum lacked these antibodies. This is consistent with our previous report[9] on the application of carbohydrate microarray technology for the serodiagnosis of melioidosis. It is evident that anti-anthrax-sera contained antibodies against both the anthrose mono- and tetrasaccharides. It is interesting to note that anthrose and the tetrasaccharide had similar qualitative (Fig. 4) spot intensities on microarrays, suggesting that anthrose was the immunological determinant. In contrast, antibodies against these saccharides were absent in the control serum. It is also clear that serum from tularemia-infected animals contained antibodies directed at the trisaccharide O-antigen. In contrast, antibodies against this saccharide were absent in the normal serum. Taken together, these results demonstrate that the carbohydrate microarray can specifically detect antibodies in the serum of individuals infected with melioidosis, anthrax or tularemia.

In our previous study,[9] we used a microarray platform by immobilizing the polysaccharides derived from the single bacterium *B. pseudomallei*, the causative agent of melioidosis. In this study, we adopted the same approach, by immobilizing 'signature polysaccharides' of *B. anthracis* and *F. tularensis*, the causative agents of anthrax and tularemia, respectively. It is clearly evident that the previous study[9] is limited to a microarray platform based on polysaccharides derived from one type of bacterium (*B. pseudomallei*). However, the present study deals with a multiplex microarray platform consisting of 'signature carbohydrates' of three different types of bacteria including *B. pseudomallei*, *B. anthracis* and *F. tularensis*. It is interesting to note that *B. pseudomallei* and *F. tularensis* are gram-negative bacteria, while *B. anthracis* is a gram-positive bacterium. Of particular importance, the present microarray platform proves that the carbohydrate microarray technology could be utilized or extrapolated for the detection of

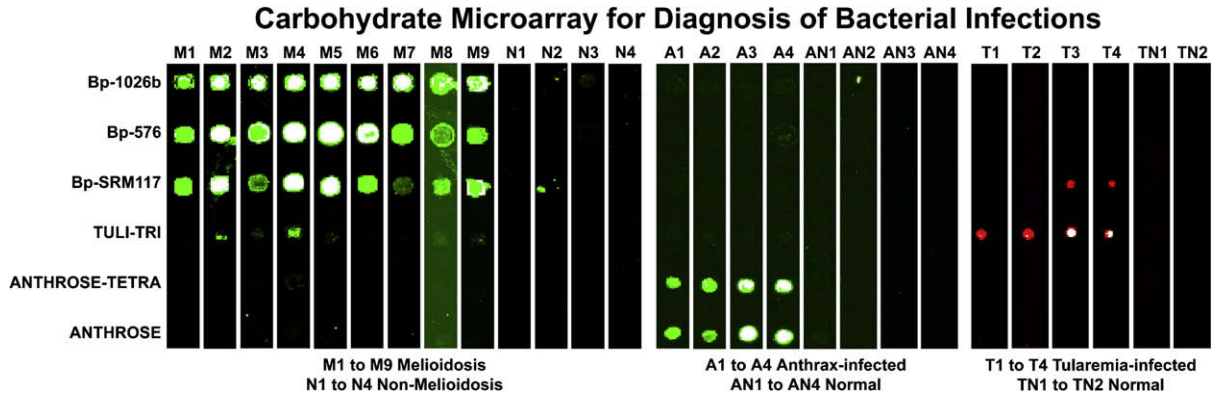

Figure 4. Typical immunoreactivity of carbohydrate microarray with anti-melioidosis human sera, anti-anthrax and anti-tularemia rabbit sera. The figure shows a comparison of microarray data for each of the 27 serum samples. Each column represents a separate array experiment involving a different serum sample. Each of the carbohydrates was printed in replicates of six on the array. However, for the sake of space, the image has been cropped to show only one spot for each carbohydrate to allow a side-by-side comparison of all 27 serum samples. Isolated polysaccharides from *B. pseudomallei*, anthrose, the anthrose-containing tetrasaccharide and trisaccharide O- antigen from *F. tularensis* were printed in replicates of six. The array was probed with different human sera and anti-tularemia rabbit sera at 1:4000 (v/v) dilution. Anti-anthrax rabbit sera was used at 1:8000 (v/v) dilution. Immunoreactivity was seen at serial dilutions from 1:1000 (v/v) to 1:128,000 (v/v) as well (diagram not shown). Anti-tularemia serum showed some immunoreactivity towards *B. pseudomallei* SRM117 capsular polysaccharide. However, estimation of spot intensity indicated that SRM117 polysaccharide intensity was <5–10%, as compared to that of *F. tularensis* trisaccharide.

serum antibodies against carbohydrate antigens specific for any gram-negative or gram-positive bacterium. The implication is that this technology could be expanded to include a variety of infectious agents.

2.4. Immunoreactivity of melioidosis, anthrax and tularemia sera

We examined the immunoreactivity of melioidosis, anthrax and tularemia sera against appropriate controls. For comparison between melioidosis versus normal and tularemia versus normal, we choose the spot intensity of microarray of polysaccharide antigen after probing with serum at 1:4000 v/v dilution,[9] as they fell on the linear portion of the curve, when plotted against a series of dilution from 1:1000 v/v up to 1:128,000 v/v. In the case of anthrax samples, a spot intensity was chosen at 1:8000 v/v serum dilution. The geometric mean of the spot intensity for each polysaccharide antigen was calculated. We compared melioidosis, anthrax and tularemia sera and their respective controls (Table 1). A non-parametric Wilcoxon test indicated that there was a significant difference in spot intensity (<0.02) between melioidosis patients and non-melioidosis with respect to 1026b, 576 and SRM117 polysaccharides. A similar significant difference (<0.05) was observed between anthrax and control sera. The difference in spot intensity (0.3) between anti-tularemia and control sera was suggestive only due to limited number of samples.

Table 1
Comparison of spot intensity[a] of carbohydrate microarray between melioidosis, anthrax, tularemia-infected versus control

Infection	Polysaccharide	Infected or immunized		Control	
		N^b	Geometric mean (SEM)	N^b	Geometric mean (SEM)
Melioidosis	Anthrose	9	311(1.3)	4	108(1.7)
	Anthrose-tetra	9	348(1.3)	4	94(1.3)
	Tuli-tri	9	926(1.4)	4	160(1.4)
	Bp-SRM117	9	18,731(1.4)	4	466(1.5)
	Bp-576	9	34,914(1.3)	4	1055(1.7)
	Bp-1026b	9	38,602(1.0)	4	427(4.8)
Anthrax	Anthrose	5	10,898(1.6)	4	70(1.2)
	Anthrose-tetra	5	11,753(1.4)	4	32(1.2)
	Tuli-tri	5	86(1.2)	4	47(1.1)
	Bp-SRM117	5	44(1.1)	4	43(1.1)
	Bp-576	5	57(1.1)	4	46(1.2)
Tularemia	Anthrose	5	87(1.5)	3	18(2.9)
	Anthrose-tetra	5	272(3.0)	3	30(2.4)
	Tuli-tri	5	11,923(1.5)	3	39(3.5)
	Bp-SRM117	5	106(2.4)	3	13(1.9)
	Bp-576	5	75(1.4)	3	6(2.6)
	Bp-1026b	5	89(1.7)	3	6(2.3)

[a] Spot intensity was geometric mean of replicates of six.
[b] The total number of individuals in the group.

2.5. Advantages of carbohydrate microarray technology over indirect hemagglutination (IHA) and microagglutination tests for the serodiagnosis of melioidosis and tularemia

Melioidosis[27] is a disease of animals and humans, which is endemic in Southeast Asia and northern Australia. Acute septicemia, pulmonary infection and subsequent acute and chronic diseases are the symptoms of melioidosis.[28] The serodiagnosis of this disease is conventionally carried out by IHA,[29] using filtered bacterial culture supernatants. Therefore, this test lacks specificity for melioidosis. This might be due to the presence of cross-reacting antibodies in other related but non-pathogenic bacteria. Therefore, we could not rule out the potential for false diagnosis. Tularemia is an infection common in wild rodents. *F. tularensis* is the causative bacterium of this infectious disease. Tularemia is transmitted to humans by contact with infected animal tissues or by ticks as one of the main vectors.[23] Tularemia cases[23] are frequently reported in the northern hemisphere, including Europe, North America, northern Russia and Japan. Possible symptoms include skin ulcers, swollen lymph nodes, fever, chills and pneumonia. The traditional serodiagnosis of tularemia is carried out by a microagglutination[30] test similar to IHA using crude bacteria, and therefore false diagnosis is possible. Furthermore, both IHA and microagglutination tests use an initial very high dilution of serum. A dilution of 1:240 v/v was generally used for the serodiagnosis of tularemia. A high dilution of 1:40 v/v and 1:60 v/v was also used for the serodiagnosis of melioidosis using IHA test. A high background and false diagnosis is possible. In the carbohydrate microarray used in this study, the starting dilution of 1:1000 v/v is used. Immunoreactivity was observed even at higher dilutions of 1:64,000 and 1:128,000 (diagram not shown). The immunoreactivity at dilutions 1:4000 and 1:8000 v/v is shown in Figure 4. The current carbohydrate microarray technology is also based on pure carbohydrate antigens and could circumvent the problem of false diagnosis.

2.6. Immunoreactivity of sera towards anthrose is specific for anthrax infection

A few reports have appeared on anthrose, a biomarker for anthrax infection, since the initial finding of Daubenspeck et al.,[21] who showed that anthrose is a specific sugar constituent of *B. anthracis* spores. Wang et al.[31] showed that rabbit IgG antibodies produced against *B. anthracis* spores specifically reacted against the tetrasaccharide containing anthrose at the upstream terminal. Similar findings were reported by Metha et al.[32] and Dhénin et al.[33] In all these assays, synthetic non-specific saccharides were used as negative controls. In our study, we did not use non-specific saccharides as negative controls. Indeed, polysaccharides or saccharides specific to each bacteria reacted strongly with their respective antibacterial sera. The other bacterial polysaccharides or saccharides served as negative controls.

2.7. Application of carbohydrate microarray technology for the diagnosis of bacterial infections

The carbohydrate microarray technology, as we outlined in our study, was tested with success for detecting *B. pseudomallei*, *B. anthracis* or *F. tularensis* antibodies in human and animal serum. We used only a small number of samples. Further validation of this technology is required by including a large number of samples in the assay. This is especially true in the diagnosis of endemic melioidosis. These bacterial organisms pose as biothreats to humans. In non-endemic settings, where biothreat issues are important, the utility of carbohydrate microarray technology for detecting antibodies to bacterial biothreat agents in serum cannot be underestimated.

3. Experimental

3.1. General methods

Polysaccharides (capsular polysaccharides and O-antigen saccharides) were prepared from *B. pseudomallei* strains (1026b, 576, SRM117) as described previously.[9] Briefly, the bacterial cell pellets were subjected to hot-phenol extraction, followed by sequential digestion with DNAse, RNAse and Proteinase K digestion followed by acetic acid hydrolysis (2% acetic acid, 100 °C). Lipid A moiety, released during hydrolysis, was removed by centrifugal filtration (Amicon centrifugal device MW cutoff 5K). A trisaccharide O-antigen of the structure α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-D-QuiNAc was purchased from Sussex Research Lab, Canada. Bacterial polysaccharides and trisaccharide were converted into glycosylamines by reductive amination in the presence of ammonium acetate, as described previously.[9] Briefly, The dry polysaccharide or trisaccharide was treated with 100 μL of 0.3 mol/L sodium cyanoborohydride in 2 mol/L ammonium acetate, pH 6.0, at 105 °C for 4 h. The reaction was terminated by the addition of 200 μL distilled deionized water and 80 μL of 6 mol/L formic acid. The contents were dried in the Speed Vac at 45 °C. Methanol (1.0 mL) was added to the reaction mixture and ammonium acetate was removed by drying in the Speed Vac at 45 °C. This procedure was repeated twice and the dry glycosylamine was dissolved in 1.0 mL distilled deionized water. Anthrose and tetrasaccharides attached to the (2-aminoethylamido)-carbonylpentyl linker were synthesized as described.[34]

3.2. Printing of carbohydrates

Super-epoxy-functionalized glass slides with 24-well chambers were purchased from CEL Associates Inc (Houston, TX). Bacterial polysaccharide glycosylamines (1 μmol/mL rhamnose equivalents) as determined by the phenol–H_2SO_4 method,[35] trisaccharide glycosylamine (1 mg/mL), the linker equipped anthrose and the tetrasaccharide (1 mg/mL) were reconstituted in Tris-buffered saline (TBS) (25 mmol/L Tris, 0.15 mol/L NaCl, pH 7.2) or in phosphate-buffered saline (PBS) (100 mmol/L phosphate, 0.15 mol/L NaCl, pH 7.2). They were printed onto Super-epoxy-functionalized glass slides using a robotic microarrayer (VIRTEK, Chip Writer Pro, Bio-Rad, Hercules, CA). Replicates of six were printed. Contact printing was carried out with 'Quill' pins (SMP3, Tele Chem International Inc., Sunnyvale, CA) resulting in spots with a diameter of 90–100 μm (dwell time, 0.2 s; humidity maintained at 60%). A similar approach of fabricating arrays that involve the printing of carbohydrate–bovine serum albumin conjugates on epoxide-functionalized glass slides was recently adopted by Manimala et al.[36] In this procedure, the amino groups of bovine serum albumin are involved in immobilization onto glass slides through covalent attachment to the functionalized epoxy groups.

3.3. Human and rabbit sera

Human serum samples were obtained on admission from unselected adult patients presenting with suspected melioidosis to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand between June and October 2004. Patients were identified during active ward surveillance, and multiple samples were taken for microbiological culture. Isolation of *B. pseudomallei* from any sample taken from a febrile patient with appropriate clinical features was considered diagnostic for melioidosis. Screened patients admitted during this period who were culture negative for *B. pseudomallei* represented non-melioidosis controls. Patients and controls lived within the province of Ubon Ratchathani.

A 10 mL blood sample was collected from each patient into a sterile 10 mL polystyrene tube with screw cap lid (Teklab Ltd, Durham, UK). The tube was centrifuged at 3000 rpm for 10 min and the serum separated and stored at −30 °C at Sappasithiprasong Hospital, before being sent on dry ice to the research laboratory at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok at the end of October 2004. Samples were maintained in Bangkok at −80 °C, thawed once to aliquot and shipped on dry ice to the USA where they were stored at −40 °C. The sera samples were inactivated by gamma-irradiation and stored at −40 °C.

Anti-spore serum was obtained by a previously published procedure.[37] The antigen was purified ungerminated spores of *B. anthracis* Ames strain, inactivated by gamma-irradiation and combined with Ribi adjuvant prior to vaccination. Rabbits were immunized with intramuscular injection of 1×10^9 spores in 1.0 mL PBS. After 4 weeks, a booster injection with the same amount of spores was given. After two weeks, rabbits were bled and serum was collected in serum tubes. Covance (Denver, PA) was involved in the immunization followed by the collection of serum. Anti-tularensis serum was obtained from New Zealand white rabbits inoculated with 1×10^6 CFU of *F. tularensis* LVS (F004)[38] initially by intravenous route followed by a booster injection after 2 weeks. Serum was collected in serum tubes. The serum samples were stored at −40 °C. The serum samples (human and rabbit) were thawed three to four times during the course of analysis, and freezing followed by thawing does not affect the immunoreactivity of the serum.

3.4. Probing of carbohydrate microarray with serum

Carbohydrate microarray slides were blocked with 10–15 μL of blocking agent, 1% casein in PBS (Pierce Biotechnology, IL) at 37 °C for 1–2 h. The slides were incubated with 10 μL of diluted serum in the blocking agent at 37 °C for 1–2 h. Serial dilutions of serum from 1:1000 v/v to 1:128,000 v/v were employed. After washing with PBS containing 0.1% Tween-20 (15 μL) five times, the slides were

incubated with 10 μL of Cy3- or Cy5-labelled appropriate secondary antibody at 1:1000 v/v dilution in the blocking agent. Incubations were carried out in a moist closed container to avoid evaporation of liquid. Cy3- and Cy5-labelled anti-rabbit IgG (H+L) were purchased from Amersham Biosciences (Piscataway, NJ). Antihuman IgG (H+L) was labelled with Cy3 bifunctional NHS (*N*-hydroxysuccinimide) ester dye using Amersham Biosciences kit. After washing, the slides were scanned for fluorescence at 532 nm (for Cy3) and at 635 nm (for Cy5) with a Gene Pix 4000B Axon scanner, using the software Gene Pix Pro 5.1. The fluorescence intensity of each spot was also measured. Even though this microarray platform is multivalent, the slide format is unidimensional,[36] as the spot intensity is a measure of fluorescence on a flat glass surface.

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