A *Burkholderia pseudomallei* protein microarray reveals serodiagnostic and cross-reactive antigens

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Understanding the way in which the immune system responds to infection is central to the development of vaccines and many diagnostics. To provide insight into this area, we fabricated a protein microarray containing 1,205 *Burkholderia pseudomallei* proteins, probed it with 88 melioidosis patient sera, and identified 170 reactive antigens. This subset of antigens was printed on a smaller array and probed with a collection of 747 individual sera derived from 10 patient groups including melioidosis patients from Northeast Thailand and Singapore, patients with different infections, healthy individuals from the USA, and from endemic and nonendemic regions of Thailand. We identified 49 antigens that are significantly more reactive in melioidosis patients than healthy people and patients with other types of bacterial infections. We also identified 59 cross-reactive antigens that are equally reactive among all groups, including healthy controls from the USA. Using these results we were able to devise a test that can classify melioidosis positive and negative individuals with sensitivity and specificity of 95% and 83%, respectively, a significant improvement over currently available diagnostic assays. Half of the reactive antigens contained a predicted signal peptide sequence and were classified as outer membrane, surface structures or secreted molecules, and an additional 20% were associated with pathogenicity, adaptation or chaperones. These results show that microarrays allow a more comprehensive analysis of the immune response on an antigen-specific, patient-specific, and population-specific basis, can identify serodiagnostic antigens, and contribute to a more detailed understanding of immunogenicity to this pathogen.

antigen discovery | melioidosis | diagnostic | antigen prediction

Understanding the interaction of the immune system with bacteria is central to an understanding of the pathogenesis of infectious disease, and also to the development of diagnostics and vaccines. Yet there is an unmet need for a comprehensive and unambiguous approach for quantifying the immune response to infection in an antigen-specific manner and on a genome-wide scale. The development of antibodies to individual components of bacterial pathogens is 1 important element of the immune response, and is especially relevant to the development of diagnostics and for therapies where antibodies play direct or indirect roles in the killing of pathogens or pathogen-infected cells. Although protein antigens are generally assumed to make up the majority of the bacterial antigens that are recognized by antibodies, the reasons why only some proteins evoke antibody responses are poorly understood. There is a general and intuitive assumption that proteins that are displayed on the surface of the bacterium are more likely to evoke an antibody response. In the case of proteins able to induce protective immunity there is some

evidence that this assumption is correct. An analysis of 72 bacterial proteins included in vaccines or shown to induce protective immunity in animal models of disease showed that 52 (72%) possessed signal sequences and were therefore likely to be located outside of the cell membrane (1). The assumption that proteins able to induce protective immunity possess signal sequences is exploited in reverse vaccinology, where candidate protective antigens are predicted from the genome sequence (2).

However, these studies fail to provide insight into the broader question of the differential abilities of bacterial proteins to induce antibody responses in a population. To a large extent, the difficulties in addressing this question are a reflection of the limitations of existing technologies for mapping the complete subset of proteins involved in the immune response against the infectious agent. These technologies rely on chromatographic or electrophoretic separation of proteins from bacteria or the screening of expression libraries. These approaches are limited by the different levels of expression of proteins in the native or recombinant hosts, specific bacteria growth conditions if cultivable, and are not conducive to high-throughput screening of a large collection of serum samples.

We have previously developed array technology that allows protein microarrays to be constructed from the predicted proteome of a microorganism (3–7). These arrays can be used to address basic questions about the interactions of a given pathogen with the host immune system (8–10), and allows the identification of proteins which can be used as diagnostic reagents or for inclusion in vaccines (3). The empirical data gathered from this type of array can also be used to evaluate and improve the accuracy of *in silico* antigen prediction of a proteome.

We report here the development of a *Burkholderia pseudomallei* protein array. *B. pseudomallei* is the causative agent of melioidosis, a serious and often fatal infectious disease of humans. It is an important medical problem across Southeast

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Asia and northern Australia, and is increasingly recognized in other tropical areas of the world including areas of South America (11, 12). The global incidence of disease is not known, but in northeast Thailand, the disease accounts for 40% of all deaths from community-acquired septicemia. The potential for the bacterium to cause disease after inhalation has also resulted in the inclusion of this pathogen on the CDC category B list of potential biological warfare and bioterrorism agents (12). Here we have used the protein array to map the antibody response in 747 serum samples from well-defined melioidosis positive and negative patients.

Results

Protein Microarray Design and Construction. We devised a protein array of 1,205 proteins, including proteins predicted to be surface located using PSORTb (13), components of the 3 different type III systems, components of the flagella, proteins identified as immunoreactive from 2D gels, and 672 proteins selected at random. This array was probed with a collection of 88 sera from melioidosis patients in Singapore (SI Text). One hundred seventy antigens with average signal intensity greater than 2.5 times the standard deviation of the average negative control spots were considered seroreactive. Of these, 80 proteins were encoded on chromosome 1, and 90 on chromosome 2. Three of the seroreactive proteins were encoded in genomic islands (BPSL1705 in GI8, BPSS0663 in GI4, and BPSS1068 in GI15), and 2 proteins were encoded in possible genomic islands (BPSS0402 and BPSL0739). Some large CDSs (coding sequences) were expressed as overlapping fragments and for many of these, we found reactivity against several of the fragments, providing evidence that epitopes are distributed throughout these proteins. For example, 6 polypeptides from the BPSS2053 CDS, encoding a 3,103 amino acid protein similar to the *Ralstonia solanacearum* probable hemagglutinin-related protein, all reacted with melioidosis sera. Four polypeptides that form the BPSS1434 CDS, encoding a 2,178 amino acid protein similar to a *Streptococcus pneumoniae* cell wall surface anchor family protein, all reacted with patient sera. Nine polypeptides from the BPSL1661 CDS, encoding a 3,229 amino acid protein similar to a *Ralstonia solanacearum* putative hemagglutinin/hemolysin-related protein, all reacted with melioidosis sera. In a previous study, 12 *B. pseudomallei* proteins were identified as immunoreactive by 2D gels (14), and all of these proteins were identified as immunoreactive using the array.

Mapping the Antigenic Profile. The seroreactive antigens were printed onto smaller arrays (Fig. 1), which we used to probe the entire collection of 747 serum samples from patients with melioidosis and control sera from individuals in Singapore, Thailand, and the USA (Table S1 and SI Text). The Singapore and Thailand melioidosis-positive and -negative cases were patients entering the hospital with symptoms of melioidosis that were later confirmed to be either positive or negative for melioidosis. The Thailand cases were from Ubon Ratchathani in Northern Thailand and healthy controls from the same region where the disease is endemic. There were also healthy controls from Southern Thailand (outside the endemic region) and patients diagnosed with leptospirosis, other bacteremia, or fungemia.

The reactivity of 747 sera from different individuals is shown as a heatmap (Fig. 2) and as a histogram (Fig. S1) with patient samples grouped according to their clinical description. Thirty-one of the most reactive serodiagnostic antigens and 31 cross reactive antigens are shown. ‘Serodiagnostic’ antigens are defined as significantly differentially reactive between the Singapore melioidosis-positive and -negative groups with Benjamini and Hochberg adjusted Cyber-T *P* values <0.05, and ‘cross-reactive’ antigens had a *P* value >0.05 (Fig. 3). Analysis of variance was performed to detect

Fig. 1. Construction of a *B. pseudomallei* Microarray. Arrays were printed containing 214 *B. pseudomallei* proteins, positive and negative control spots. The arrays were read in a laser confocal scanner, analyzed, and the data normalized as described in the *Materials and Methods* section. Protein expression efficiency was determined to be 99.2% by probing against a carboxy-terminal HA tag for quality control. Each array contains positive control spots printed from 4 serial dilutions of human IgG (HMIg), and the intensity of these spots was similar for both serum samples. Each array also contained 6 “No DNA” negative control spots, and the reactivity of these spots was low for both serum samples. There are also 4 serially diluted EBNA1 (EBNA) protein control spots that are reactive to varying degrees in different subjects, as expected, and provide a methodological control. The remaining spots on the array are in vitro transcription/translation reactions expressing 183 different *B. pseudomallei* proteins that were selected from our primary array analysis. The signal intensity of each antigen is represented by rainbow palette of blue, green, red, and white by increasing signal intensity. Representative microarray immunofluorescence images of individual patient sera are displayed.

differences in signal intensity between groups. For the serodiagnostic antigens there is a significant difference when comparing melioidosis-positive patients to all other groups ($P = 3.6 \times 10^{-7}$), but the Singapore and Thailand melioidosis patients are not different from each other ($P = 0.55$), and all melioidosis negative patients are not different from each other ($P = 0.91$) (Fig. S1). All of the sera react similarly to the cross-reactive antigens whether from melioidosis-positive or -negative individuals, healthy subjects from endemic or nonendemic areas, or patients with other infections (P value = 0.08).

Determining a Set of Serodiagnostic Antigens. There were 2 distinct collections of melioidosis-positive and -negative specimens used for this work, from different clinical investigators and clinical sites in Thailand and Singapore, and different definitions of the cases and controls were used in each location. The incidence of melioidosis is relatively low in Singapore, and exposure to *B. pseudomallei* is considered to be an infrequent event. The Singaporean cases were clinically confirmed melioidosis and positive by the indirect hemagglutination assay (IHA) and about 40% of these samples were proven culture positive. All of the controls for this collection lacked clinical signs of melioidosis disease and were also IHA negative. The Northeast Thailand collection is from a region where the disease is considered endemic. All of the cases are proven culture positive, and the controls are culture negative but IHA positive subjects are not excluded, because it is known that healthy individuals in the general population in Northeast Thailand can be IHA positive. For all of the work reported here we used the Singaporean cases and controls to develop a classifier that can distinguish melioidosis-positive and -negative subjects, and tested sensitivity and specificity of the classifier independently on the Thailand collection.

Our goal was to establish a collection of antigens that could be used as a multiplex set to accurately distinguish the melioidosis cases from controls. As such, we studied the discriminatory power of different sets of ORFs using receiver operating characteristic (ROC) curves. First, ROC curves were generated for individual serodiagnostic antigens to assess their ability to separate the control and disease cases. The serodiagnostic

The classifier threshold that yielded the highest accuracy in the Singapore data set was then used to predict the samples in the

Improving Serodiagnostic Accuracy. To test the feasibility of using the serodiagnostic antigens on an alternative analytical platform, 10 serodiagnostic proteins were printed onto an Optitran nitrocellulose membrane (typically used for western blots) using a BioDot jet dispenser (*SI Text*). These 10 serodiagnostic proteins were chosen for being highly significant and highly seroreactive in the protein microarray assay. The paper was then cut into 3 mm strips to produce ‘immunostrips’ (also called ‘line blots’) (Fig. 4B). The individual strips were probed with 60 different melioidosis positive sera and 67 melioidosis negative sera from the Singapore collection. Reactive bands were visualized after incubation with alkaline phosphatase conjugated anti-human secondary antibody followed by substrate, and the band intensities quantified with ImageJ (17). Melioidosis patients reacted strongly against the serodiagnostic antigens although the intensity pattern varied depending on the patient. Naïve and healthy control subjects had low reactivity against these serodiagnostic antigens.

Functional Classification of the Reactive Antigens. We next classified the serodiagnostic and cross-reactive antigens according to annotated and computationally predicted features. The complete analysis is in [Table S4](#) and a summary in Table 1. Annotated features were taken from the Artemis database on the Sanger website which has 2 categories of functional definitions called ‘Colour’ and ‘Class.’ Each of the 13 colors has a functional definition, and each of the 5,853 proteins in the database is assigned exactly 1 color. The results in Table 1 show that only 2 of the 13 color classifications are significantly

The majority of these serodiagnostic antigens (36/49) were encoded on chromosome 2 of *B. pseudomallei* K96243. (Table S2) Chromosome 2 has previously been suggested to be associated with genes required for adaptation and survival in different niches (18), and our finding that serodiagnostic antigens were preferentially encoded on chromosome 2 is consistent with this hypothesis. The serodiagnostic proteins included a range of known or putative virulence associated proteins including components of TTSS3 (BPSS1532, BipB and BPSS1525, BopE) (19–21) and a type IV pilus protein PilO (BPSS1599) (22).

Table 1. Enrichment table

	Counts		Cross-reactive					Serodiagnostic		
	Whole proteome	Large chip	Hits		Fold enrich	P value	Hits	Fold enrich	P value	
Artemis 'colour' definitions										
pathogenicity/adaption/chaperones	368	106	13	*	2.50	1.2E-03	11	*	2.55	2.4E-03
Surface (outer membrane, inner membrane, secreted, surface structures)	1533	352	31	*	1.80	1.8E-04	25	*	1.75	1.1E-03
All other categories	3952	747	15		—	—	13		—	—
Total	5853	1205	59				49			
Artemis 'class' definitions										
Chaperones	30	6	0		0.00	1.0E +00	3	*	12.30	1.2E-03
Membrane/exported/lipoproteins	397	174	15	*	1.76	2.1E-02	12		1.70	5.8E-02
Outer membrane	81	48	2		0.85	1.0E +00	9	*	4.61	7.2E-05
Computational predictions										
PSORTb cytoplasmic	1975	293	2	*	0.14	1.6E-05	7		0.59	1.2E-01
PSORTb cytoplasmicmembrane	1054	94	11	*	2.39	4.3E-03	5		1.31	5.8E-01
PSORTb extracellular	37	11	5	*	9.28	8.8E-05	3	*	6.71	8.3E-03
PSORTb outermembrane	150	71	10	*	2.88	1.6E-03	14	*	4.85	2.2E-07
PSORTb periplasmic	130	30	5	*	3.40	1.3E-02	2		1.64	3.5E-01
PSORTb unknown	2507	706	26	*	0.75	2.2E-02	18	*	0.63	1.8E-03
SignalP >= 0.7	1041	340	40	*	2.40	1.1E-10	30	*	2.16	9.7E-07

We have also interfaced our dataset with data on genes which are up-regulated in the hamster model of melioidosis (23). Genes encoding 6 of the 31 diagnostic signature proteins (BPSS0476; GroES: BPSL1445; putative lipoprotein BPSL2017; Di-heme cytochrome c peroxidase; BPSL2697; GroEL: BPSL3319; flagellin; and BPSL2520: putative exported protein) were up-regulated in vivo. Chaperones and stress response proteins from a range of pathogens have frequently been found to react with the appropriate convalescent sera (14, 24–28). Both GroEL and DNA K have previously been identified as reactive with sera from melioidosis patients (14, 25).

Discussion

This study is the most extensive use of a diverse collection of patient sera on a protein microarray. Of 1,205 proteins probed, 49 antigens were identified that are significantly more reactive in patients with melioidosis and 59 antigens were cross-reactive in healthy people and patients with other infections. Because melioidosis is confined to relatively small localized regions of the world, it might have been anticipated that immunoreactivity against this species would be localized as well. Our finding that these cross-reactive antigens also reacted equally with sera from individuals in the USA suggests that cross-reactive antibodies may be responsible for these signals. This finding contrasts markedly to our previous studies with *Francisella tularensis* (7, 29), *Borrelia burgdorferi* (5), *Plasmodium falciparum* (6), and vaccinia (8), where reactivity of arrayed proteins with naïve patient sera was minimal or not seen.

The antibodies in melioidosis negative sera may be indicative of past exposure to bacteria related to *B. pseudomallei* which possess similar proteins. For example, BPSS0542 (glycosyl hydrolase), BPSL1207 (polyribonucleotide nucleotidyltransferase), and BPSS2136 (family S43 nonpeptidase homologue) are identical in a range of *Burkholderia* species such as *Burkholderia thailandensis*, *B. vietnamiensis*, and *B. cenocepacia*. *B. thailandensis* is found in many regions of the world including SE Asia and the USA (30) and the *B. pseudomallei* and *B. thailandensis* genomes are highly syntenic (31). Bacteria of the *B. cepacia* complex are also widely distributed worldwide and are frequently the cause of complications in cystic fibrosis. All of these bacteria rarely cause disease in healthy individuals but it is possible that subclinical disease results in the induction of an immune response to these bacteria and the development of

antibodies that react with the protein array. Even though cross-reactivity is widespread, some individuals within any region were essentially immunologically naïve. The results reported here suggest widespread exposure and cross-reactivity to nonpathogenic *Burkholderia* sp., but these conclusions warrant corroborating data from additional endemic and nonendemic regions and comparative data on arrays from nonpathogenic strains.

The information from the array was used to devise an accurate prototype diagnostic test, using differentially reactive antigens while avoiding the cross-reactive ones. The microarray platform may not necessary be the most sensitive to produce the most accurate test results. Serodiagnostic antigens discovered by microarray were transferred to the immunostrip platform, the Singaporean specimen collection was used to train the classifier, and the classifier tested on the Thailand collection. The immunostrip test allowed for the correct identification of 95% of melioidosis-positive samples and 83% of melioidosis-negative samples, a major improvement over the most commonly used diagnostic test (the IHA test) which identifies 79% and 72% of positive and negative samples, respectively. We regard the microarray as a serodiagnostic antigen discovery platform. Antigens discovered by microarray can be transferred to other more sensitive platforms to give more accurate test results.

The data we report here also provide a broader insight into the antigens that are recognized by the immune system after natural infection. Reactivity was not evenly distributed across the proteome and the cross-reactive and serodiagnostic antigens are selectively enriched with proteins from specific functional categories. Membrane and secreted molecules accounted for 52% of the reactive antigens, but this class of molecule constitutes only 29% of the proteins on the array. Similarly, pathogenicity-island related proteins and chaperones (as annotated in the Artemis database) accounted for 22% of the reactive antigens but only 9% of the proteins printed on the large array were from this category. Three of the 6 annotated chaperones printed on the array were serologically reactive, and all 3 of these were serodiagnostic for melioidosis. PSORTb predicts 82 proteins (7% of the large array) to be extracellular or outmembrane localized, and 32 (39%) are antigenic. Proteins with a signal peptide are enriched on the reactive antigen lists and proteins lacking a signal peptide sequence are underrepresented. Proteins involved in central and intermediary metabolism are also significantly underrepresented on the reactive antigen list. These

examples illustrate that molecular recognition by the immune system is not stochastic. There is a preference for recognition of surface molecules, chaperones, and pathogenicity-related factors, and the PSORTb outer membrane and extracellular computational tool predicts antigenicity. These results classifying antigenicity by protein function are not out of line with expectations but they are far more quantitative and informative than previously reported.

Seventy-four percent of the antigens fall into functional annotation categories that are enriching features, but the other 26% of the reactive antigens are from categories with proteomic features that are underrepresented on the immunoreactive antigen list. We have not yet identified predictive features of these molecules that selectively target them for immune recognition. Some of the molecules with proteomic features that are underrepresented on the hit list may be expressed at high levels in vivo making them targets for immune recognition independent of their functional category. Although certain protein categories are enriched in the immunoreactive antigen list, there is no category that is entirely reactive. For example, there are 458 proteins on the array in the pathogenicity and outer membrane categories, but only 17% of these molecules are reactive. Part of the explanation for nonreactivity may be that the proteins expressed in the cell free in vitro expression system are not in the proper conformation to be recognized by the antibodies. For example, we have shown that proteins requiring disulfide bonds for their native conformation may not be seen unless in vitro expression is carried out under oxidizing conditions (4). Lack of posttranslational modification, incomplete folding of proteins expressed in vitro, or unfolding of proteins bound to nitrocellulose are other factors that could result in an underestimate of the total number of reactive antigens determined by this method, but our experience with other agents indicates that this effect is actually marginal (4). At this point, any in silico algorithm for

predicting the antigenic profile based on sequence data alone will be imperfect because the majority of proteins predicted are nonreactive. Furthermore, there are categories of reactive molecules with proteomic features that are not enriched in the antigenic profile, so they would not be predicted. The data presented here highlight the need to improve in silico prediction algorithms for antigen discovery and vaccine development.

These results provide significant insight into the antigenicity of a bacterial pathogen, while both confirming and overturning assumptions that have previously been made about the nature of immunoreactive proteins. In addition to contributing to a more profound understanding of the humoral immune response to infection, this work could lead to the development more accurate tests for clinical diagnosis and for worldwide disease surveillance.

Materials and Methods

Protein Microarray Chip Fabrication and Probing Methods. Protein microarray chips consisting of 1,205 *B. pseudomallei* antigens were fabricated as described previously (SI Text) (3, 4, 7).

SI. The description of the serum samples, immunostrip fabrication and probing, and data analysis methods are in the SI Text.

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