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Distinct human antibody response to the biological warfare agent *Burkholderia mallei*

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The genetic similarity between *Burkholderia mallei* (glanders) and *Burkholderia pseudomallei* (melioidosis) had led to the general assumption that pathogenesis of each bacterium would be similar. In 2000, the first human case of glanders in North America since 1945 was reported in a microbiology laboratory worker. Leveraging the availability of pre-exposure sera for this individual and employing the same well-characterized protein array platform that has been previously used to study a large cohort of melioidosis patients in southeast Asia, we describe the antibody response in a human with glanders. Analysis of 156 peptides present on the array revealed antibodies against 17 peptides with a > 2-fold increase in this infection. Unexpectedly, when the glanders data were compared with a previous data set from *B. pseudomallei* infections, there were only two highly increased antibodies shared between these two infections. These findings have implications in the diagnosis and treatment of *B. mallei* and *B. pseudomallei* infections.

Burkholderia mallei and the closely related *Burkholderia pseudomallei* are CDC Category B Bioterrorism Agents due to the history of confirmed use of *B. mallei* in biological warfare including the US Civil War,[1] World War I,[2] World War II[1,3] and purportedly in Afghanistan in the 1980s.[4] *B. mallei* is an obligate pathogen of horses that causes glanders, a chronic disease known since the time of Aristotle,[1] that can infect humans who work in close proximity to infected animals.[1,5] In this work we employ a protein microarray, which was previously used in the study of a large cohort of patients in southeast Asia with *B. pseudomallei* infections,[6] to analyze the targets of antibodies produced against *B. mallei* in the first human case of glanders in the US since 1946.[7,8] This work provides the first direct comparison of the human antibody reaction against *B. mallei* and against *B. pseudomallei*. Importantly, despite the high level of similarity between *B. mallei* and *B. pseudomallei* and the similarity in disease presentation, the antibody profiles are strikingly different. This suggests that different therapeutic approaches might be required for each infection and also provides potential antigens for the development of a practical differential diagnosis approach.

Glanders has been eradicated from most of Europe and all of North America through aggressive infection control programs.[1] As a result little is known about *Burkholderia mallei* pathogenesis in humans compared with *Burkholderia pseudomallei*, a related environmental bacterium and opportunistic pathogen, endemic in southeast Asia.[5]

In 2000, a worker at United States Army Medical Research Institute of Infectious Diseases (USAMRIID) accidentally acquired a *B. mallei* infection.[8] This case has been the subject of previous reports due in part to the unique opportunity to study a human glanders infection for which pre-exposure and post-exposure serum exists.[9,10] A recent analysis indicated that levels of *B. mallei*-specific IgA, IgG and IgM were highly elevated at 64 d post-infection,[10] but the immunogenic antigens were not identified. We employed a previously described *B. pseudomallei* protein array[6,11] to perform an in-depth analysis of this serum. This array was previously used to identify antibodies produced against *B. pseudomallei* in a cohort of melioidosis patients in southeast Asia.[6,11] The protein microarray incorporates 214 *B. pseudomallei* K96243 computationally-predicted antigenic peptides, and construction of this array was previously described.[6] The *B. mallei* genome is a reduced version of the *B. pseudomallei* genome that has 99.1% identity for shared genes and does not contain additional genes.[5] Accordingly, the *B. pseudomallei* protein microarray can be used to detect reactivity to *B. mallei* proteins as 156 of the peptides are present in some form in both species (**Table S1**).[12,13] Microarrays were hybridized using pre-exposure serum and serum from 2 mo after symptoms manifested in the researcher who had contracted glanders.[8] Hybridization, image scanning, and data acquisition were performed as previously described.[6] Data were analyzed by generating $\log_2$ ratios of (post-exposure intensity/pre-exposure intensity).

When compared with the pre-exposure serum, the $\log_2$ ratio of post-exposure to pre-exposure intensities were > 2 for 7 out of 156 peptides present on the array and between 1 and 2 for 12 additional peptides (**Table 1**; **Table S1**), indicating increased production of antibodies targeting these antigens. Some of the peptides above the cut-off level that are not actually encoded

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Table 1. Highly increased antibodies reactivity in a human glanders infection

Log_2 (post-exposure/ pre-exposure)	B. pseudomallei locus	B. mallei locus	Product name	Presence in human melioidosis sera[§]
10.47	BPSL1925	BMA1071*	Hypothetical protein	
10.41	BPSS1401	BMAA1630[†]	Type III secretion-associated protein	
3.43	BPSL2520	BMA0434	Hypothetical protein	Recovered patients and healthy controls
3.28	BPSS1620	BMAA1630[†]	Type III secretion protein	
2.24	BPSL2697	BMA2001[‡]	Chaperonin GroEL	Recovered patients and healthy controls
2.11	BPSL3222	BMA2642	50S ribosomal protein L7/L12	Recovered patients only
2.08	BPSS0477	BMA2001[‡]	60 kDa chaperonin	Recovered patients and healthy controls
1.96	BPSL2919	BMA2431	10 kDa chaperonin	
1.63	BPSL2698	BMA2002	Co-chaperonin GroES	
1.24	BPSL0999	BMA0711	Putative OmpA family transmembrane protein	
1.15	BPSS2136	BMAA0356	Family S43 non-peptidase homolog	
1.09	BPSL1937	BMA1088	Lipoprotein	
1.09	BPSS0943	BMAA1286	Porin protein	
1.07	BPSS1390	BMAA1602	Type III secretion system protein	
1.03	BPSS1534	BMAA1532	Type III secretion protein	
1.01	BPSS1532	BMAA1530	Type III secretion system cell invasion protein	Recovered patients and healthy controls
1.01	BPSS0783	BMAA0633	Outer membrane porin protein	
1.00	BPSL2756	BMA2073	Minor Type 4 pilin	
0.97	BPSS1599	BMAA1609	Type 4 pilus biosynthesis protein	Recovered patients only

*DNA between BMA1070 and BMA1072 is 99% identical to BPSL1925, but gene not annotated. [†]BMAA1630 corresponds to BPSS1620, cross-reaction possible due to very high identity to BPSS1401. [‡]BMA2001 corresponds to BPSL2697, cross-reaction possible due to very high identity to BPSS0477. [§]As reported by Suwannasaen et al.[11]

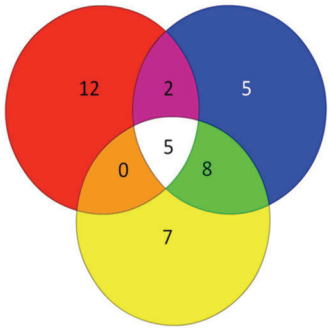
within the *B. mallei* genome were detected by the array. However, as discussed by Waag et al.,[10] prior to working at USAMRIID the subject had worked with both *B. pseudomallei* and *B. mallei* and thus may have elevated levels of antibodies to some peptides due to previous exposures.

Antibodies against five different type III secretion system components were highly increased in the human glanders infection (**Table 1**), four of which (BPSS1390/BMAA1602, BPSS1401/BPSS1620/BMAA1630 and BPSS1534/BMAA1532) were not seen in melioidosis serum from patients in southeast Asia.[11] BPSS1532/BMAA1530 was seen in the glanders infection as well as in melioidosis patients and healthy controls from southeast Asia (**Table 2**). The type III secretion system is a bacterial protein export mechanism that forms syringe-like appendages present in several bacterial species that function to inject effector molecules into host cells. The type III secretion system has been shown to be required for virulence in mouse and hamster models for *B. mallei* and *B. pseudomallei* (reviewed by Galyov et al.[5]).

Type 4 pili are complex structures used by Gram-negative and Gram-positive bacteria for motility and surface attachment.[14] The *B. mallei* major pilin, PilA, and *B. pseudomallei* minor pilin, PilV, have both been shown to be immunogenic, but failed to protect mice against challenge.[15,16] We noted antibodies against PilA (BPSL0782/BMA0278) were increased slightly in the serum from the glanders infection (**Table S1**), while antibodies against a different minor pilin of the Type 4 pili system (BPSL2756/BMA2073) were increased 2-fold (**Table 1**); PilV (BPSS1593) was not represented on the array. Antibodies against the Type 4 pilus component BPSS1599/BMAA1609 were detected in human glanders serum and serum from recovered melioidosis patients[11] (**Table 1**).

In addition to antibodies to recognized virulence factors, antibodies against a lipoprotein (BPSS1937/BMA1088), three porins/outer-membrane proteins (BPSL0999/BMA0711, BPSS0943/BMAA1286 and BPSS0783/BMAA0633) and four chaperonins (BPSL2697/BPSS0477/BMA2001, BPSL2919/BMA2431 and BPSL2698/BMA2002) were strongly increased above background (**Table 1**). While little is known about the role of lipoproteins in *B. mallei* and

Table 2. Comparison of the antibody profiles of serum from human glanders, recovered melioidosis patients and healthy controls from southeast Asia



B. pseudomallei locus	*B. mallei* locus	Product name
BPSL0280	BMA3335	Flagellar hook-associated protein
BPSL0999	BMA0711	Putative OmpA family transmembrane protein
BPSL1445	BMA1416	Putative lipoprotein
BPSL1465	BMA1397	Peptidase
BPSL1661	NA	Putative hemolysin-related protein
BPSL1901	BMA1042	Putative membrane protein
BPSL1902	BMA1043	Putative membrane protein
BPSL1925	BMA1071	Hypothetical protein
BPSL1937	BMA1088	Lipoprotein
BPSL2063	BMA0840	Putative membrane protein
BPSL2096	BMA1487	Putative hydroperoxide reductase
BPSL2520	BMA0434	Hypothetical protein
BPSL2522	BMA0436	Outer membrane protein A (OmpA) precursor
BPSL2697	BMA2001	Chaperonin GroEL
BPSL2698	BMA2002	Co-chaperonin GroES
BPSL2756	BMA2073	Minor Type 4 pilin
BPSL2765	BMA2082	Putative OmpA family lipoprotein
BPSL2919	BMA2431	10 kDa chaperonin
BPSL3222	BMA2642	50S ribosomal protein L7/L12
BPSL3319	BMA2873	Flagellin
BPSL3398	BMA2955	ATP synthase alpha chain
BPSS0477	BMA2001	60 kDa chaperonin
BPSS0734	BMAA1932	Outer membrane efflux protein
BPSS0783	BMAA0633	Outer membrane porin protein
BPSS0943	BMAA1286	Porin protein
BPSS1390	BMAA1602	Type III secretion system protein
BPSS1401	BMAA1630	Type III secretion-associated protein
BPSS1434	NA	Putative membrane-anchored cell surface protein
BPSS1492	BMAA0749	Hypothetical protein Bim A
BPSS1512	BMAA0729	Type VI secretion protein, TssM
BPSS1532	BMAA1530	Type III secretion system cell invasion protein
BPSS1534	BMAA1532	Type III secretion protein
BPSS1588	BMAA1597.1	Putative exported protein BPSS1588
BPSS1599	BMAA1609	Type 4 pilus biosynthesis protein
BPSS1620	BMAA1630	Type III secretion protein
BPSS1974	BMAA0090	Putative lipoprotein
BPSS2053	NA	Putative cell surface protein
BPSS2136	BMAA0356	Family S43 non-peptidase homolog
BPSS2141	BMAA0351	Periplasmic oligopeptide-binding protein precursor (OppA)

Red, human glanders; blue, recovered melioidosis patients;[11] yellow, healthy controls from southeast Asia.[11] ORFs in the accompanying list are color coded to match the Venn diagram. NA, gene not present in *B. mallei*.

B. pseudomallei pathogenesis, two lipoproteins not represented on this array have been identified in previous *B. pseudomallei* studies: a signature-tagged mutagenesis experiment identified BPSL3147 (BMA2723) as being required for virulence in mice,[17] while immunization with BPSL2151 (BMA1547) was shown to provide protection from, but not clearance of, *B. pseudomallei* in mice.[18] Porins and outer-membrane proteins have been characterized in membrane preparations of *B. mallei* and *B. pseudomallei*.[19] However, only one of the porins (BPSS2136/BMAA0356) that reacted at elevated levels with the human glanders serum (**Table 1**) was detected as one of the top 20 proteins in *B. mallei* outer membrane preparations.[19] A second protein, BPSS0943/BMAA1286, was also detected in the outer membrane preparations[19] and highly elevated in the human glanders infection (**Table 1**). These data suggest that not all outer membrane proteins in *B. mallei* are equally antigenic.

Using this serum, Amemiya and colleagues previously identified via ELISA that IgG against GroEL increased ~10-fold and anti-DnaK IgG increased ~1.5-fold.[9] The present study showed that antibodies against GroEL (BPSL2697/BMA2001) were increased ~4.8-fold while antibodies against DnaK (BPSL2827/BMA2326) were increased ~1.4-fold (**Table 1**; **Table S1**). The difference in observed levels of antibodies against GroEL may reflect sensitivity differences between the technologies.

Due to limited data available for *B. mallei* infections, it is impossible to evaluate these results in the context of existing literature without the obvious comparisons to the genetically related *B. pseudomallei*. The distinctive antibody profile from this glanders infection compared with existing melioidosis literature suggests some interesting contrasts between the pathogenesis of these two diseases. A recent study used the same protein array platform to probe antibody response in individuals who had recovered from melioidosis in southeast Asia.[11] We noted some overlap between the antibodies identified in recovered patients and healthy controls and those from recovered patients with the results from the human glanders infection (**Tables 1 and 2**). Interestingly, there was only minor overlap between the antibody reactivity found in the glanders serum and that from the melioidosis patients. Only antibodies against BPSS1599 (Type 4 pilus biosynthesis protein) and BPSL3222 (50S ribosomal protein L7/L12) were elevated in both infections and not present in serum from healthy humans in southeast Asia (**Tables 1 and 2**).

Differences were noted in antibodies produced from this human infection and reports on antibodies from horses with glanders. Using phage display technology, Tiyawisutsri et al. screened equine glanders infection serum and identified antibodies against four chromosomal loci that were over-represented in their library.[20] Two of these four loci encoded a total of three peptides present on the array (BMAA1324, BMA1024 and BMA1027), but none of them had greatly increased antibodies in the human infection (**Table S1**). The reason for these differences is not known, but it could be due to the different screening technology, the fact that horses are prone to a chronic glanders infection while the human case was acute and/or different immunogenic antigens that are prominent in these different hosts.[5,8]

These data can also be compared with the outer membrane proteome of *B. mallei*.[19] The general absence of proteins identified by screening for the presence of, and increase in, antibodies compared with the proteome data[19] is intriguing as it would be anticipated that highly expressed proteins would overlap with the proteins that elicited the strongest antibody response. These data suggest that while proteins may be highly expressed in vitro, they are either not highly expressed in vivo or may be non-immunogenic.

As this approach has shown promise and greatly expands on existing research, it warrants further studies using an animal model so that proper statistical analyses and comparisons may be performed. This will also allow for a comparison between host data in order to verify that antibodies produced in mouse infections are representative of antibodies produced in a human infection. In other studies protection in mice can be achieved with monoclonal antibodies against *B. mallei* administered prior to, but not after, challenge.[21] However, in these studies the animals' spleens were heavily colonized with *B. mallei* despite surviving the infection[21] and a similar result was observed with a lipoprotein vaccination of *B. pseudomallei*.[18] This current work presents data and identifies potential immunogenic antigens that may be exploited to develop new protective antibodies that overcome this limitation.

As the report by Waag et al. shows,[10] serum from this individual reacted to killed whole cells of *B. mallei* and *B. pseudomallei*. While that approach allows for serodiagnosis of exposure, it is non-specific. Having a detailed comparison will greatly aid in the development of serodiagnostic antibodies for *B. mallei* and *B. pseudomallei* infections. When these human glanders results were compared with serum from recovering melioidosis patients and healthy controls from southeast Asia[11] there were 12 antibodies that were highly increased only in the glanders infection while five were highly present only in the melioidosis samples (**Table 2**). Additionally, seven antibodies were highly present only in the healthy controls while five were detected for all three conditions (**Table 2**). Using a *Yersinia pestis* protein microarray, Keasey et al. showed that cross reactive antibodies are generated to proteins from number of Gram-negative pathogens, including *B. mallei* and *B. pseudomallei*.[22] However, the only protein that was cross-reactive and common between the protein microarray used in our study and the *Y. pestis* protein microarray was GroEL. This result suggests that the 12 proteins which generated antibodies found only in glanders serum represent candidate antigens for the differentiation of glanders and melioidosis infections in humans and that these also have less risk for cross-reactivity with other pathogens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/virulence/article/22056

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