Protein Microarrays for Characterisation of the *Bacillus anthracis* 'infectome'

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Introduction

Bacillus anthracis is a Gram-positive spore-forming, rod-shaped bacterium that is the etiological agent of the zoonotic disease Anthrax. Reservoirs for anthrax are wild and domestic grass-eating animals, most commonly sheep, goats and cattle and their proximity to humans within an agricultural setting can cause infection and disease. Disease may also be contracted through either direct contact with infected animals or through contact with animal by-products such as wool, skins or bone meal, where spores can survive in the environment for decades. Disease can occur when spores enter the body through breaks in the skin, via ingestion or by inhalation and has a number of clinical presentations. The Anthrax bacillus expresses a number of proteins responsible for the pathology of disease, including the protein toxins lethal factor (LF), oedema factor (OA) and protective antigen (PA). Other proteins are likely to be expressed during infection, which may contribute to the disease process.

Bacterial pathogens express a large number of proteins during the course of infection, many of which are non-specific house-keeping proteins. However a proportion of expressed proteins are specifically associated with pathogenicity the '*infectome'* and are expressed predominantly during the course of infection. Many of these general eubacterial and pathogen-specific proteins will be recognised as 'non-self' by the host immune system and an immune response mounted against them, by the 'humoral' arm of the immune system, which confers long-term 'memory' or 'protective' immunity. This provides protection against challenge with the same or similar pathogens upon re-exposure. Antibody responses are a key feature of the protective immune response and antibody reactivity against pathogen antigens is one of the key immunological tools used in diagnosis of infectious disease. This can be used for identification of immuno-dominant antigens or protein epitopes which are useful for conferring protective immunity and can also be used for development of more focused diagnostic tools and identification of new vaccine candidates.

We have used this approach for identification of immuno-dominant Anthrax antigens using a commercial whole-genome, expressed open-reading frame (ORF) protein microarray. Interrogation with sera from a number of sources has enabled characterisation of the profile of proteins recognised by individuals exposed to live Anthrax or proteins and identification of unique proteins with further potential for diagnostic development.

Method

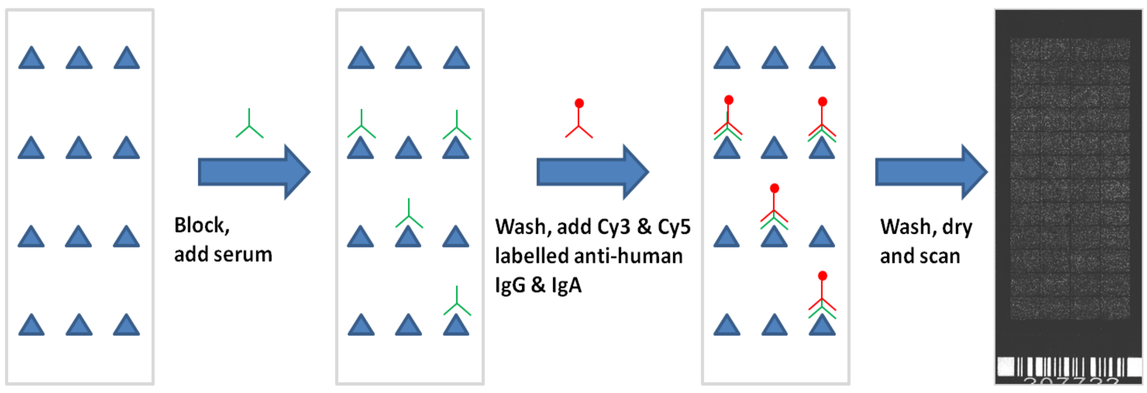


Figure 1. Diagram of microarray hybridisation method

Sera were hybridised to the Anthrax protein microarray from volunteer donors who had received the UK AVP Anthrax protein vaccine, occupationally exposed workers from a Belgian wool-sorters factory (pulmonary exposure), naturally infected Turkish individuals (confirmed cutaneous anthrax) and naive controls. Immuno-reactive protein features were identified though secondary hybridisation with Cy3 & Cy5-labelled anti-human Ig conjugates (***Figure 1***) and were screened for both IgG and IgA-immunoreactive antigens.

Arrays were then scanned using a Molecular Devices GenepixPRO 4200A scanner and the raw data analysed using the quantification software BlueFuse which logs the fluorescence intensity of each spot on the array. The data were normalised to the global median, fused by name and then analysed further using analysis of variance (ANOVA) and heteroscedastic (unequal variance), two-tailed student T-tests between all sample groups (n=6 in each group).

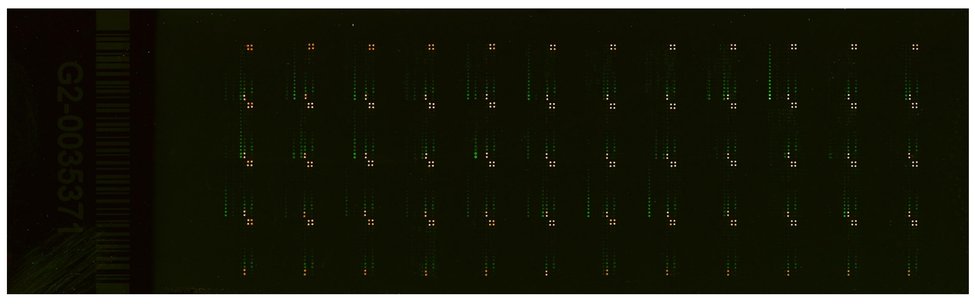


Figure 2. Image of a scanned Invitrogen ProtoArray hybridised with human vaccinee sera and probed with Cy3 & Cy5-labelled goat anti-human IgG & IgA.

Results & Discussion

Hybridisation of sera to the Anthrax whole ORF protein array revealed a profile of immunoreactive protein features in each sample (***Figure 2).*** Through statistical analyses we have identified a number of significant proteins which are recognised specifically by individual groups i.e. naturally exposed/infected individuals/vaccinees or are shared between groups. Many proteins were recognised by most sera tested, including controls and in the main were found to be ubiquitous proteins found in all eubacteria e.g. ribosomal proteins. These may represent common proteins recognised by residual immune memory from previous bacterial infections. Other proteins were identified which are specific to the Bacillus cereus group, of which *B. anthracis* is a member, but were not *B. anthracis* specific. A small number of protein biomarkers were identified as specific to *B. anthracis*.

Many of these immuno-dominant antigens are not recognised by vaccinated or individuals, suggesting that there are proteins associated with live *bacilli* that are not currently found in the AVP. These analyses have revealed interesting details concerning the number of antigens recognised by individual test groups and between groups. Detailed comparison between the infected, occupationally challenged, vaccinated and control groups (***Figure 3***) has revealed that there are a large number of immune-dominant antigens recognised by naturally exposed individuals, particularly in the Turkish cutaneous infected group that are not recognised by the control or vaccinated groups. This suggests there are a significant number of antigens expressed during natural anthrax infection that are not found in other commonly encountered bacteria, found in un-germinated or early germination-stage spores or are present in the current UK AV vaccine. This provides an interesting insight into the protein expression patterns or '*infectome*' of virulent *B. anthracis* in a host infection environment.

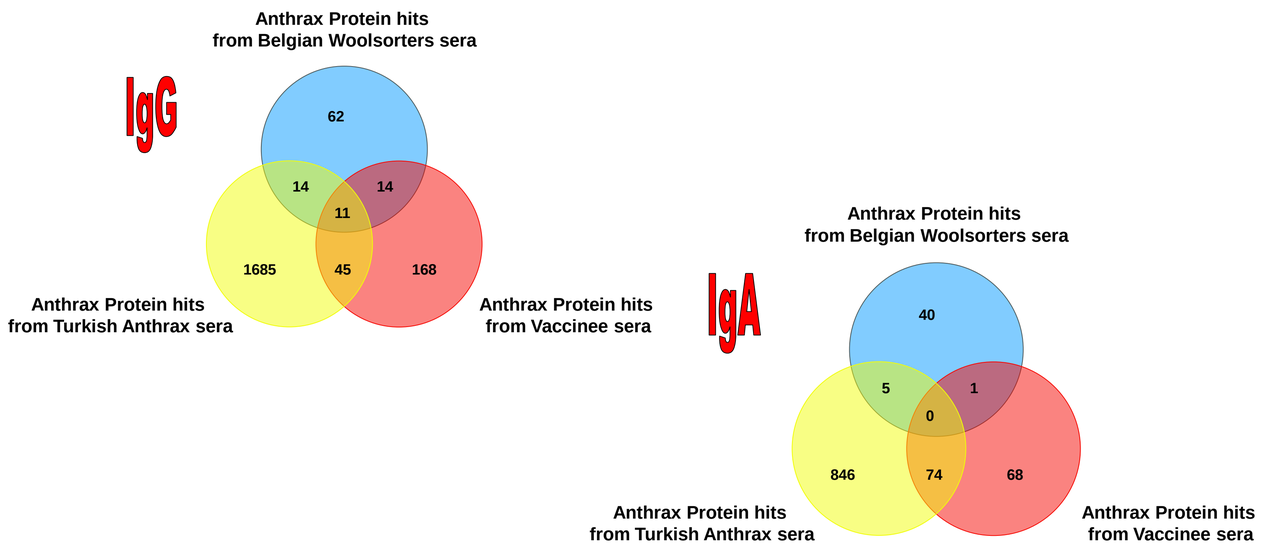
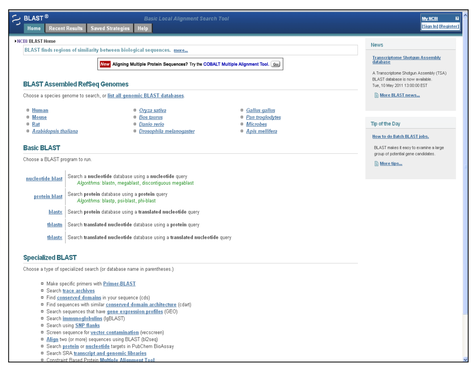


Figure 3. Venn diagrams of statistically significant hits and their distribution between test groups. Data shown was derived from two-tailed, two-sample heteroscedastic student T-tests on signal intensity over and above unchallenged controls.



Each significant protein hit with a P value < 0.000063342484 (from ANOVA analysis) was compared against the NCBI database using TBLASTN (***Figure 4***) to identify unique *B. anthracis* protein biomarkers. 16 significant protein hits were found specific *to B. anthracis* which have not been documented in previous publications.

Figure 4. http://blast.ncbi.nlm.nih.gov/Blast. cgi

These identified proteins may only be expressed during the infection process and could indicate up-regulation of alternative pathways during *B. anthracis*-host interactions. These new proteins show promise for ongoing analysis to confirm their growth phase specificity and their suitability for further development. These could make highly attractive candidates for diagnostic and vaccine biomarker development as they are likely to be specifically associated with expression or alternative virulence pathways during the infectious phase of the pathogen.

Summary

- Protein arrays are a useful tool for identifying immuno-reactive proteins
- These studies provide an interesting insight into the transcriptome of virulent *B. anthracis* in a host infection environment;
- We have found that there are many more antigens recognised by individuals with cutaneous anthrax than recognised by the other representative groups
- These studies have identified previously unidentified Anthrax antigens which may be connected with the infection process
- Identified protein biomarkers may be useful for further analysis and potential commercial development.