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Pre-symptomatic Prediction of Illness in Mice Inoculated with Cowpox

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Abstract. We describe here research directed towards early (presyndromic) diagnosis of infection. By using a mouse model and a multi-component blood protein diagnostic tool we detected cowpox infection several days in advance of overt symptoms with high accuracy. We provide details of the experimental design and measurement technique and elaborate on the long-range implication of these results.

Keywords. Biodefense, disease, diagnostic, mouse model, pox

1. Introduction

There is growing urgency to develop techniques for rapid detection and diagnosis of infectious disease in human populations. Rapid detection is critical for reducing the morbidity and mortality from either bio-terrorism events or newly emerging diseases. Current methods for direct agent detection using culture methods or microbial component detection using antibodies or PCR have a number of limitations. Rapid microbial detection in blood may not be possible for agents that remain localized to the site of infection or agents that do not appear in peripheral blood until the later stages of the disease. Cell culture and sample enrichment procedures can also require several days. Finally, newly emerging diseases or genetically modified organisms may have never been seen before complicating organism-specific detection methods.

Host responses may provide early signals in blood even from localized infections. Cells in the innate immune system produce a rapid response after initial contact with a potential pathogen. While pathogen responses initially involve local cell signaling processes designed to activate near-by immune cells, cascades of cytokines and chemokines are released into the periphery to activate additional cells types and to cause them to migrate to the site of the infection. Thus, early immune responses may provide general indicators for the presence of many different infection types. A spectrum of innate and adaptive immune markers, in combination with other biochemical markers comprise a 'signature' that may allow for timely, disease-specific detection.

Direct studies of the time course of natural diseases in humans is complicated by the difficulty in defining exposure doses and exposure timing. Model systems, both in cell culture and animal models, allow precise control over exposure dose and timing. Studies of specific tissue types in culture have been used to define early responses in cells at the to pathogen exposure, but it is often difficult to relate these tissue-specific responses to systemic responses in the whole animal. Consequently, we chose cowpox

infection in mice as the model system for studies of early disease detection through blood protein signatures. Mice have been shown to be susceptible to infection by both cowpox and vaccinia viruses [1, 2]. The severity of infection varies from mild to lethal depending on the strain of mouse, strain of virus, and the location and dose of viral challenge. Viral instillation in the lung of mice produces a pulmonary infection that has been used as a model for pulmonary smallpox infection in humans, so that this model has been used extensively for studies of anti-viral drugs [3, 4].

The TK- strain of cowpox virus into BALB/c mice model used for these studies exhibits three major features important to early detection of infections. First, there is an incubation period of about 6 days before the mice show signs of illness. This provides the opportunity to assess protein changes in serum throughout this prodromal period, as well as the period of active infection. Second, this model produces a localized infection in the lung, with no live virus detectable in the blood using plate assays. Thus, this model is well suited to analyze whole-animal systemic responses in blood to a localized infection. Finally, the conditions used for these studies produce a serious illness, but no lethality was observed from viral infection. This feature allows assessment of the early stages of recovery from infection, and insures that the biochemical changes we observe reflect moderate disease rather than the severe toxicity of super-lethal doses.

In ths paper we describe the use of canonical variate analysis (CVA) to predict pre-symptomatic illness in mice inoculated with cowpox virus. Signatures were developed from measurements of specific blood serum protein concentrations determined by Rules Based Medicine, Inc., Austin, TX using Luminex Liquid Array LA technology. We applied CVA to training sets of data to find transformations to the canonical space specifying the optimal group separation. These transformations were then applied to test sets to determine where individual mice mapped to in canonical space. Group membership of each individual was predicted by assigning membership to the training group lying closest to the individual in canonical space. Finally, assigned membership was compared with known true membership and the results expressed as a confusion matrix.

We have made three separate analyses and have recommendations for follow on analyses based on the results for the two experiments analyzed here.

2. Experiments

All experimental work with the mice was performed in a biosafety level 3 (BSL-3) animal facility at the University of New Mexico [5, 6]. Mice were infected with CPV by surgical intratracheal instillation. The infected mouse group received 50 ul of media (PBS 2.5% BGS diluted 1:2 in Tris buffer) containing CPV. The sham group received the same intratracheal surgical procedure with 50ul of media only. The naïve control group received no surgery or CPV.

Animals were sacrificed at each experimental time point to obtain serum samples. The animals were euthanized via Avertin overdose. Blood was collected from clipped vena cava and placed in eppendorf tube. The blood was allowed to coagulate for at least 30 minutes at 4°C. The whole blood was then centrifuged at 4500 RPM for 10 min in microfuge, and the serum from each mouse was placed in individual eppendorf tubes. The samples were stored at -80°C and analyzed at the end of the study. Serum volumes varied from 700 to 200 microliters, with lower recoveries at the peak of the disease.

The two experiments analyzed herein were conducted a month apart in spring-summer of 2005. In each experiment 60 mice were given tracheotomies. In 30 of these mice, cowpox virus (CPV) was injected in the tracheotomies. In the other 30 mice, saline solutions were injected (sham). On days 2, 3, 4, 5, 6, and 8, five CPV and five sham mice were sacrificed and blood collected from the chest cavities. Mouse serum samples were transported frozen on dry ice to Rules Based Medicine (RBM) for multiplex immunoassay analysis (Rules Based Medicine, Inc., Austin, TX). RBM utilizes a multi-analyte panel to quantify the concentrations of about 60 host antigens in mouse serum samples. A total of 50 microliters of serum were shipped for each analysis, with all serum samples coded for blind analysis. While some aspects of this multiplex analysis are proprietary, the following briefly describes the approach. Sample is incubated with a mixture of fluorescently labeled microsphere types, with each bead type conjugated to a different capture antibody. A mixture of biotinylated secondary antibodies is then added to label bead-captured antigen. Finally streptavidin-phycoerythrin is added to fluorescently label the captured antigen. Flow cytometric analysis with a Luminex 100 flow analyzer is used to quantify fluorescence signals for ach antigen in the analysis. Purified antigen standards are included is some samples to develop standard curves for relating bead-based fluorescence to antigen concentration. Sample processing typically requires 1 to 3 hours, and flow cytometric analysis takes about 1 minute per sample [7, 8, 9]. Additional details of the RBM analysis can be found at www.rulesbasedmedicine.com .

Assay results are reported in units of protein concentration. A "Lowest Detectable Dose" (LDD) is also reported for each antigen. The LDD is the antigen concentration that produces a fluorescence signal that is 3 standard deviations above the fluorescence of negative control beads. Table 1 below lists the 54 protein for which we had assays, of these we used 43 proteins in the analysis described herein. These 43 proteins are the ones for which at least two mice had levels above the LDD in both experiments. Protein concentration values were set to 0.5*LLD for assay results that were below the LDD for the protein.

Animals were monitored for behavioral signs of illness including ruffled fur and decreased activity. While a few animals began to show symptoms on day 5, most animals showed clear signs of illness on days 6 and 8.

Table 1. Proteins measured in the RBM panel used for experiments 1 and 2.

Apo A1 (Apolipoprotein A1)	IL-1alpha (Interleukin-1alpha)	MDC (Macrophage-Derived Chemokine)
CRP (C Reactive Protein)	IL-1beta (Interleukin-1beta)	MIP-1alpha (Macrophage Inflammatory Protein-1alpha)
EGF (Epidermal Growth Factor)	IL-2 (Interleukin-2)	MIP-1beta (Macrophage Inflammatory Protein-1beta)
Endothelin-1	IL-3 (Interleukin-3)	MIP-1gamma (Macrophage Inflammatory Protein-1gamma)
Eotaxin	IL-4 (Interleukin-4)	MIP-2 (Macrophage Inflammatory Protein-2)
Factor VII	IL-5 (Interleukin-5)	MIP-3beta (Macrophage Inflammatory Protein-3beta)
FGF-9 (Fibroblast Growth Factor-9)	IL-6 (Interleukin-6)	Myoglobin
FGF-basic (Fibroblast Growth Factor-basic)	IL-7 (Interleukin-7)	OSM (Oncostatin M)

GCP-2 (Granulocyte Chemotactic Protein-2)	Insulin	RANTES (Regulation Upon Activation, Normal T-Cell Expressed and Secreted)
GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	IP-10 (Inducible Protein-10)	SCF (Stem Cell Factor)
Haptoglobin	KC/GROalpha (Melanoma Growth Stimulatory Activity Protein)	SGOT (Serum Glutamic-Oxaloacetic Transaminase)
IFN-gamma (Interferon-gamma)	Leptin	TIMP-1 (Tissue Inhibitor of Metalloproteinase Type-1)
IgA (Immunoglobulin A)	LIF (Leukemia Inhibitory Factor)	Tissue Factor
IL-10 (Interleukin-10)	Lymphotactin	TNF-alpha (Tumor Necrosis Factor-alpha)
IL-11 (Interleukin-11)	MCP-1 (Monocyte Chemoattractant Protein-1)	TPO (Thrombopoietin)
IL-12p70 (Interleukin-12p70)	MCP-3 (Monocyte Chemoattractant Protein-3)	VCAM-1 (Vascular Cell Adhesion Molecule-1)
IL-17 (Interleukin-17)	MCP-5 (Monocyte Chemoattractant Protein-5)	VEGF (Vascular Endothelial Cell Growth Factor)
IL-18 (Interleukin-18)	M-CSF (Macrophage-Colony Stimulating Factor)	vWF (von Willebrand Factor)

3. Data Analysis

To analyze the training sets we used standard CVA as discussed in Krzanowski and Srivatava [10, 11]. These analyses were done in a backward elimination selection method, which proceeds using the test on the change in Wilks ratio for each elimination as proposed by Rao [12] and described in detail by Hawkins and McHenry [13, 14]. See detail on signature development and class prediction in Appendix I.

The data was analyzed by considering the CPV mice sacrificed on days 2 and 3 as one group, the CPV mice for days 4 and 5 as another group, the CPV mice for days 6 and 8 as a third group, the sham mice for days 2 and 3 as a fourth group, the sham mice for days 4 and 5 as the fifth group, and the sham mice for days 6 and 8 as the sixth group. After the training set is analyzed to find the positions of the training mice (and groups) in canonical space, the "unknown" individuals from training set are then assigned to one of these six groups by mapping them to canonical space and finding the nearest training group for each test individual.

We have made three separate analyses. First we used experiment 1 (the first experiment performed) as a training set and experiment 2 as the test set. Second we used experiment 2 as the training set and experiment 1 as the test set. Third we made a 10-fold external cross validation on both experiments. In this cross validation method, we combine both experiments into one data set. Thus there will be the same six groups but each group will have twenty members instead of the ten when the experiments are considered separately. Then the combined data set is repeatedly reanalyzed by random partitioning followed by training and testing. This method provides an estimate of the overall error rate in predicting class membership. In our case assigning class

membership is equivalent to deciding whether or not an individual mouse is infected with CPV and how much time has elapsed since the infection (or tracheotomy in the case of sham mice).

Details on the class prediction and cross validation techniques are given in Appendix I and II.

4. Results

Training Set: = Experiment 1 / Test Set = Experiment 2

In this analysis we found that eight proteins significantly separated the six groups in the training set. The proteins were:

Proteins used in CVA of experiment 1	
MCP-5_(Monocyte_Chemoattr	KC_GROalpha_(Melanoma_Gro
Haptoglobin	IL-1alpha_(Interleukin-1a
MIP-1beta_(Macrophage_Inf	Myoglobin
IL-18_(Interleukin-18)	MMP-9_(Matrix_Metalloprot

The canonical transformations were constructed from the concentrations of these eight proteins.

In Table 2 we show the confusion matrix for using experiment 1 as the training set and using experiment 2 as the test set. To read the table read each row from left to right. We see immediately that while pox mice for days 4-5 and days 6-8 were well predicted to be the appropriate pox group, sham mice were overwhelmingly predicted to be pox mice. This level of false positives renders this particular transformation useless. The variables (proteins) that separated the groups in experiment 1 were peculiar to experiment 1, possibly due to some irreproducible idiosyncrasy of the conditions under which experiment 1 was conducted.

Table 2. Confusion matrix for training CVA using experiment 1. Group assignments made on experiment 2. Column labeled by "Total" contains the number of mice in groups labeled in column one labeled by "Group"

Group	**Total**	**Sham 2-3 days**	**Sham 4-5 days**	**Sham 6-8 days**	**Pox 2-3 days**	**Pox 4-5 days**	**Pox 6-8 days**
Sham 2-3 days	10	0	0	0	4	6	0
Sham 4-5 days	10	0	0	0	1	9	0
Sham 6-8 days	10	0	0	3	1	5	1
Pox 2-3 days	10	0	0	0	4	6	0
Pox 4-5 days	10	0	0	0	0	9	1
Pox 6-8 days	10	0	0	0	0	1	9

Training Set = Experiment 2 / Test Set = Experiment 1

In this analysis we found that eight proteins significantly separated the six groups in the training set. The proteins were:

Proteins used in CVA of experiment 2	
MIP-1beta_(Macrophage_Inf	Haptoglobin
IP-10_(Inducible_Protein-	GCP-2_(Granulocyte_Chemot
CD40_Ligand	Leptin
MCP-5_(Monocyte_Chemoattr	Growth_Hormone

The canonical transformations were constructed from the concentrations of these eight proteins. Note that there is some overlap with the proteins chosen in the first training analysis. There is also some discrepancy between the two sets.

In Table 3 we show the confusion matrix for using experiment 2 as the training set and using experiment 1 as the test set. To read the table read each row from left to right. We see immediately that sham mice are overwhelmingly mapped to sham mice. We see that pox mice in the 4-5 day group are mainly mapped to the correct pox group and pox mice in the 6-8 day groups are mainly mapped to pox mice. We did not do well predicting the exact group for 2-3 day pox mice.

Table 3. Confusion matrix for training CVA using experiment 2. Group assignments made on experiment 1. Column labeled by "Total" contains the number of mice in groups labeled in column one labeled by "Group".

Group	**Total**	**Sham 2-3 days**	**Sham 4-5 days**	**Sham 6-8 days**	**Pox 2-3 days**	**Pox 4-5 days**	**Pox 6-8 days**
Sham 2-3 days	10	5	3	0	2	0	0
Sham 4-5 days	10	2	1	3	4	0	0
Sham 6-8 days	10	2	5	3	0	0	0
Pox 2-3 days	10	2	1	0	7	0	0
Pox 4-5 days	10	0	0	1	1	8	0
Pox 6-8 days	10	0	0	0	2	0	8

We regard the above results as extremely promising as most pox mice in the 4-5 day group are correctly mapped, even though most animals show no signs of illness at this time period.

Cross Validation

In the 10-fold external cross validation we found that seventeen proteins were selected as significantly separating the six groups.

In Table 4 we show the confusion matrix for the cross validation. Shams are mapped mainly to shams. We see that pox mice in the 4-5 day group and in the 6-8 day group are mainly mapped to the correct group of pox mice. We did not do well predicting the exact group for 2-3 day pox mice. Many 2-3 day pox mice are mapped to 2-3 day sham mice. While most pre-symptomatic animals at 4-5 days can be correctly classified using these 17 proteins, protein alterations at 2-3 days may be too small to differentiate pox from sham animals.

These results show promise for disease detection when one considers that which sham-group a sham mouse belongs to is of little concern as long as we know it is a sham mouse. However which groups a pox mouse belongs to is of concern because of treatment options (in other diseases) or public health considerations.

Table 4. Cross validation confusion matrix

Group	Total	Sham 2-3 days	Sham 4-5 days	Sham 6-8 days	Pox 2-3 days	Pox 4-5 days	Pox 6-8 days
Sham 2-3 days	20000	12639	2087	1398	3245	631	0
Sham 4-5 days	20000	2974	9658	6534	833	1	0
Sham 6-8 days	20000	332	8161	10185	771	0	551
Pox 2-3 days	20000	6462	155	93	13289	0	1
Pox 4-5 days	20000	161	123	124	1762	17597	233
Pox 6-8 days	20000	17	1	0	1746	144	18092

5. Conclusion

In spite of the aggressively invasive nature of the inoculation, we were able to see the pattern of expressed proteins relax to normal then signal an alert to infection prior to symptoms. This gives promise for the development of a cost effective triage diagnostic that would optimize response to a disease epidemic or pandemic. The next step in the research is to compare a second disease to the cowpox protein signature and confirm that selectivity is possible.

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Appendix I. Class Prediction by Canonical Analysis

Denote the number of groups as h, the number of observations as N, and the number of variables as p. Divide the data set into a training set and a test set with observation numbers N_r and N_S, respectively, and $N=N_r+N_S$. To perform class prediction on a test set using canonical analysis, first form the between-group sum-of-squares-and-cross-products matrix (SSCP), denoted by **B**, and the within-group SSCP **W** from the training-set data matrix. Then solve $\mathbf{Be} - \lambda\mathbf{We}=0$ for the eigenvalue λ and the eigenvector **e**. There will be $\min(p, h-1)$ nonzero eigenvalues. The associated eigenvectors are the only ones we are concerned with. These eigenvectors map both the training set and test set observations to canonical space, i.e., the position of an observation on the axis defined by the ith eigenvector is $y_i=\mathbf{e}_i^{\mathbf{T}}\mathbf{x}_j$ where **T** denotes the transpose and $\mathbf{x}_j$ denotes the vector in RBM protein measurements of a particular observation j. All the xj's from a particular group in the training set are used to define the centroids (mean position of the kth group is $\boldsymbol{\mu}_k$) of the group in canonical space. Each $\mathbf{x}_j$ from the test set is assigned membership in the group whose centroid is nearest to it in canonical space, i.e., **y** belongs to the kth group if $\|\mathbf{y} - \boldsymbol{\mu}_k\|$ is the minimum for $\|\mathbf{y}-\boldsymbol{\mu}_i\|$ for all i.

In the backward elimination procedure, we begin with p proteins and eliminate the protein contributing the least to the discrimination. This continues successively until only one protein is left. At each stage we calculate the canonical analysis and assign membership of the test set observations. So at each stage we have the number of incorrect assignments for that number of proteins used in the canonical analysis.

Appendix II. 10-Fold External Cross Validation and Data Handling

Cross Validation

In an M-fold external cross validation, the data set is partitioned into M subsets of approximately equal size. Each subset is selected in turn as the test set, the remaining M–1 subsets are combined as the training set. Then the discrimination method is applied to the training set and any variable selection is carried out for that training set. After the discrimination function is constructed, we use the discrimination function to assign group membership to the observations in the test set. This procedure is repeated for all the M subsets. The distinction between an external cross validation and an internal cross validation is that in the former case variable selection occurs each time a discrimination function is constructed. In the latter case, variable selection is done only once at the outset and then the same variables are used for all construction of discrimination functions. Because the full data set is partitioned randomly into the M subsets, one must perform the portioning many times to get a sense of the statistical variation in the error rate of group assignment. In the cases studied here, we performed 1000 partitions. Ambroise and MacLachlan [15] have established the value of the 10-fold external cross validation to estimate of the error rate.