

## **Pre-Symptomatic Diagnostic Method for Infection by Pathogens**

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### **Abstract**

This project examined the use of chemical analysis of expired breath as a means for early detection of pulmonary inflammation such as would accompany infection by pathogens. Laboratory rats were exposed to a pulmonary irritant, and then samples of expired breath were collected and analyzed by coupled gas chromatography and mass spectrometry over the next 24 hours. Statistical comparison of the spectra from exposed and unexposed (control) animals suggested some differences in the two populations, but robust signatures have not yet been identified. More data analysis needs to be done.

### **Introduction**

A major need in public health nowadays is improved rapid diagnostic methods for determining as soon as possible if a person is about to become sick in response to exposure to some kind of pathological agent. If this determination can be made really early – before classical symptoms appear – then effective treatment can begin immediately. Furthermore, persons who are not about to become sick can be dismissed quickly from the health care facilities and the existing resources can be focused more effectively on those who need the care.

Specific examples of the situation described above include the anthrax exposures in the fall of 2001 when many people were given antibiotic treatment as a precaution because it was not practical to determine if these people had been exposed or not. The spread of flu and other epidemic illnesses could be contained and health care resources could be managed much more effectively if it were practical to identify infected persons before classical symptoms occur.

A common pathway of infection is via the lungs. As infectious agents take hold in the lungs and begin to infect the body, it is plausible to presume that there will be early chemical changes in the expired breath that can be observed and correlated with the infection. The goal of this project has been to find such pre-symptomatic signatures of infection. If signatures can be found, then attention can be given to developing effective diagnostic tests to detect these specific signatures using easily available samples of expired breath.

### **Technical Approach**

Laboratory rats were exposed to aerosolized endotoxin (a pulmonary irritant) for a short time while confined in a chamber designed for such exposure. Expired breath was collected from the animals before exposure and again after exposure at 1, 5, and 24 hours. Control (unexposed) animals were also used and handled in exactly the same manner as the exposed animals. The expired breath samples were analyzed

by gas chromatography and mass spectrometry to obtain a comprehensive detailed characterization of the chemical composition of the gas. Then robust statistical analysis tools were used to look for differences in the chemical composition of the expired breath from exposed animals and unexposed (control) animals.

All work with laboratory animals was done at the University of Tennessee Medical Center, Knoxville, in collaboration with Dr. Michael Karlstad under protocols approved by the Animal Care and Use Committees at the University of Tennessee and ORNL. Laboratory rats were obtained from a commercial supplier, acclimated for a few days in the animal facility, and then fasted for 24 hours in preparation for experiments. Animals were trained briefly for confinement in exposure chambers (see Figure 1) prior to initiation of the experiments.

A four-chambered apparatus specifically designed for exposure of laboratory rats to aerosolized materials and collection of expired breath (Figure 1) was procured from CH Technologies, Westwood, New Jersey. The apparatus was maintained at a constant temperature of 30 °C. Medical-grade air was purged through the system at a constant flow rate to sustain the animals. The gas was cleaned first by passing it through an organics trap containing activated carbon and a hygroscopic gel for removal of water vapor. Lipopolysaccharide endotoxin was obtained from Sigma-Aldrich, dissolved in deionized water, and placed in the nebulizer at the bottom of the apparatus.

Expired breath samples were collected from each chamber into mylar gas sample bags. Typically about five liters of expired breath was collected from each animal at each sample event by sampling for ten minutes. Gas bags were transported to ORNL for the chemical analyses, which were performed later the same day (or the next day in some cases in order to manage sample load).

Baseline expired breath samples were collected from each animal prior to exposure to aerosolized endotoxin or aerosolized deionized water (control animals). Then the exposure was conducted for 30 minutes. At the end of the exposure period, the animals were removed from the confinement chambers and returned to standard animal cages until time for the next expired breath sample (1, 5, and 24 hours post-exposure). Not surprisingly, animals defecated and urinated frequently in the confinement chambers, and undoubtedly the expired breath samples contain some volatiles associated with these products. However, it is expected that these effects are distributed uniformly throughout the data and will not correlate with exposure to endotoxin. Animals and chambers were cleaned whenever the animals were removed in order to minimize the effects.

Thirty-two animals were used in this study. Twenty were exposed to endotoxin, and 12 were control animals exposed only to aerosolized water in place of endotoxin. Each animal was handled in exactly the same manner insofar as possible. After the expired breath samples were obtained, the animals were euthanized according to approved procedures. Lungs were lavaged, the populations of neutrophils were determined, and other physiological and histological observations were made to assess the extent of irritation resulting from the endotoxin.

Coupled gas chromatography / mass spectroscopy (GC/MS) was used to characterize the chemical composition of the expired breath. A commercial GC/MS system was equipped with a cryogenic trap (liquid nitrogen) to condense volatiles from approximately 200 mL of gas pulled through the trap with a small vacuum pump. The cryogenic trap was located in the oven chamber of the GC. Following two minutes of sampling through the cryogenic trap, a temperature program was run from 35 to 210 °C over 43 minutes with a 50 meter, DB-5 phase column. The ion-trap MS generated m/z spectra once per second, and the spectra were stored electronically.

The GC and MS spectra were then analyzed using statistical tools. Each complete spectrum (the results from each gas sample) contained 2600 elements of retention-time data from the GC, and each retention

time element contained 450 elements of  $m/z$  values from the ion-trap MS. Very early and very late data elements were observed to be very noisy and variable (not surprisingly), and only the midranges were used for robust statistical analysis.

Chemical analysis spectra for the 32 animals at pre-exposure (baseline) and 1, 5, and 24 hours post-exposure were compared with each other in detail. The basic approach was to use one animal as a reference for comparison to all the other animals in turn. Differences were calculated at individual locations in the spectra and normalized by the local variance (an approximation of the Mahalanobis distance). The feature regions exhibiting the greatest differences between exposed and control animals were located by the computational algorithm and then visualized using scatter plots.

## Results

No robust differentiation of the exposed and control animals could be found anywhere throughout the data. Four of the statistically best separations at 24 hours are shown in Figure 2. It can be seen that the separation is not sufficient to be useful for distinguishing exposed and control animals. The results were basically the same (no distinct separation) regardless of which animal was used as the reference animal. Results for the 1- and 5-hour data were similar to those for the 24-hour data.

An alternate means to visualize the data and look for distinguishing characteristics was to compare the clustering of the exposed animals as a group (i.e., their variability compared to each other) and the clustering of the control animals as a group. It was found that in general, the clustering in the control animals was significantly better (tighter, less variation) than in the exposed animals.

The comparison of clustering in the 1-hour post-exposure data is shown visually in Figures 3 and 4 in which the ratios of the variations within the two groups are plotted as a function of GC retention time. The upper chart in each figure is the ratio of the variation in the exposed group to the variation in the control group, and the lower chart in each figure is the inverse ratio. (Note that Figure 3 covers the window from 16 to 26 minutes in the GC retention time, Figure 4 covers 16 to 43 minutes, and the vertical scales are the same within each figure but are different for the two figures.) Large spikes in the top charts indicate regions in the data where the exposed animals are noticeably more variable than the control animals group. Only in the window from 25 to 30 minutes (approx) is the variation in the control group slightly greater than in the exposed group. Similar qualitative patterns are seen in the 24-hour data. The 5-hour data show less relative scatter (more uniformity). The qualitative trends are the same regardless of what animal is used as the reference animal. Specific chemical compounds that are contributing to the observed variability have not been identified yet.

However, the relevance of these trends for diagnosing exposure is dubious because the baseline data show the same trend before any exposure to anything. That is, when the baseline expired breath samples for the 12 animals that will be controls and the 20 that will be exposed are compared as two populations, the to-be-exposed animals scatter more than do the to-be-control animals. The explanation for this observation remains a mystery; no factors are known that would differentiate the animals prior to exposure.

It was necessary to use a reference animal for these comparisons because no means was readily available to identify specific features (peaks) as specific chemical compounds. With more effort and resources, the latter approach could be employed to allow much better alignment of the data among animals, which would remove some of the uncertainty in the data. It is conceivable that this approach might lead to discovery of significant difference features that are not detectable by the methods used to date.

Counts of cell types in the bronchoalveolar lavage fluid indicated significant rises in neutrophils following exposure to endotoxin. Neutrophils were typically 3 to 5 percent in unexposed animals and increased to over 40 percent after exposure. Thus it appears that significant pulmonary inflammation was produced.

### **Summary and Conclusions**

Although the populations of exposed and control animals exhibit some apparent differences in the extent of variation within each population, these patterns also appear in the baseline data before the animals are exposed. This latter result is totally unexpected, and no explanation has been found yet. Furthermore, no specific markers or signature compounds have been identified yet that distinguish exposed animals from control animals. Efforts will continue to better understand the data and utilize more powerful data analysis methods. The quantity of data is huge, and data mining efforts will continue.

The variability in scatter described previously, if validated, might be developed into a diagnostic method. (The mystery of the baseline data would have to be resolved, too.) For example, perhaps it could be established that certain features of expired breath from a healthy animal (or human patient) fall within a narrow range, whereas these features often lie outside this narrow range for an exposed animal (patient). The results from this present study suggest that lying “outside the normal range” means both higher than and lower than the normal range instead of always higher or always lower. A significant drawback to this higher *and* lower pattern is that some exposed individuals may fall within the normal range and thus will be false negatives. However, it may be possible to minimize such false negatives by looking at multiple features in the composition of the expired breath.

The NIADS/NIH has expressed interest in this work previously (contingent on preliminary results), and the project has been recognized by the OSTP in their broad scope of technical activities being reviewed for the new Department of Homeland Security. If sufficiently robust evidence for signatures can be found in the data, proposals will be submitted to these sponsors and others.

### **Publications Derived from this Project**

None to date.

### **Intellectual Property Derived from this Project**

None to date.



Figure 1. Experimental apparatus for exposing laboratory rats to aerosolized endotoxin and collecting expired breath in mylar bags.

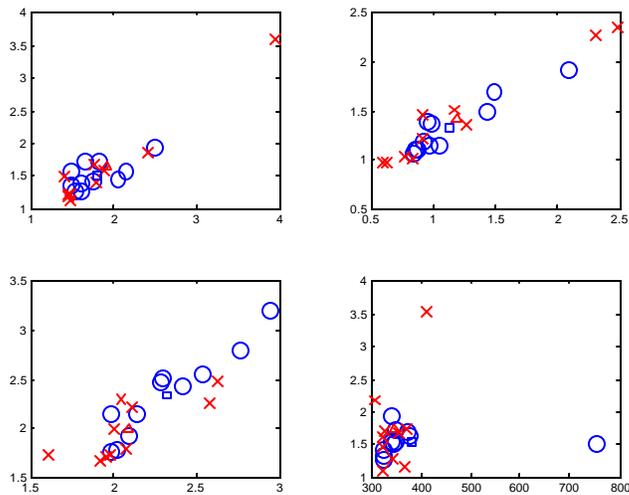


Figure 2. Four best pair-wise scatter plots using animal 21 (exposed) as reference at 24 hours post-exposure. Crosses are exposed animals and circles are control animals. Note that the two sets of animals are not well separated.

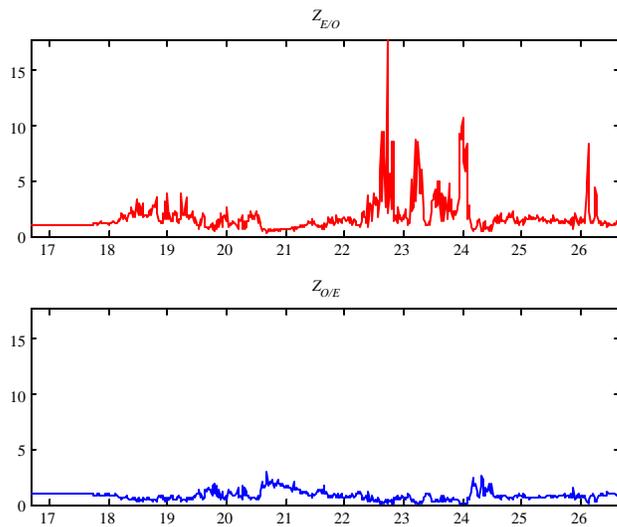


Figure 3. One-hour post-exposure scatter ratios in the 16 to 26 minute window with animal 10 (exposed) as the reference. Top chart is ratio of exposed group to control group; bottom chart is ratio of control group to exposed group.

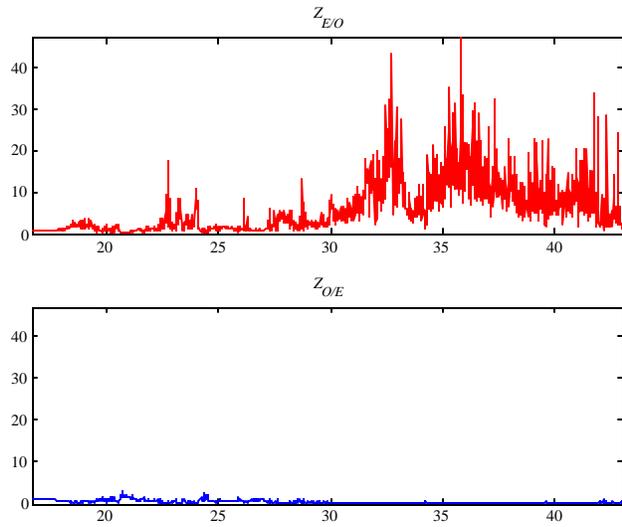


Figure 4. One-hour post-exposure scatter ratios in the 16 to 43 minute window with animal 10 (exposed) as the reference. Top chart is ratio of exposed group to control group; bottom chart is ratio of control group to exposed group.