TESTING THE VIABILITY OF STORED FROZEN SERUM SAMPLES FROM THE AIR FORCE HEALTH STUDY USING HUMAN MULTI-ANALYTE PROFILES (MAP™)

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Introduction

The Air Force Health Study (AFHS) is a 20-year prospective study of the health, mortality and reproductive outcomes of Ranch Hand veterans who participated in the aerial spraying of herbicides, including Agent Orange contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin), in Vietnam from 1962 to 1971. The comparison group consisted of Air Force veterans who flew transport missions in Southeast Asia but were not involved in spraying herbicides. Six comprehensive medical examinations were conducted between 1982 and 2002 collecting a wealth of data and biological samples. Over 80,000 biological specimens including serum, blood, adipose tissue and urine were collected and stored during the course of the study, with some specimens stored for over 24 years. The present study examines whether the stored serum samples are well preserved and could be used for further research using microsphere-based multiplexed immunoassays - Human Multi-Analyte Profiles (MAP™) by Rules-Based Medicine (RBM; Austin, TX).

Materials and Methods

The details of study design and subject selection were published previously.[1] The study included periodic comprehensive medical examinations and in-person interviews, conducted in 1982, 1985, 1987, 1992, 1997, and 2002. To be selected for the viability study each participant had to fulfill the following criteria: a) be fully compliant to all six physical examinations, b) have in storage multiple 10 ml frozen serum specimens collected at each of the first five physical examinations, c) not have a history of cancer, d) have at least one quantifiable dioxin measurement, and e) signed an informed consent form. From participants satisfying all five criteria (n=988), five AFHS veterans were randomly chosen. One 10ml serum sample per examination per participant was selected for a total of 25 serum samples. The selected specimens were packed on dry ice and shipped to the contracted laboratory. Laboratory personnel were blinded to personal identifying information, cohort participation or dioxin levels of the selected participants.

The Rules-Based Medicine's Human MAP™ was used to analyze samples. MAPs are high-density, quantitative immunoassay panels that allow identification of biomarker patterns and may provide a comprehensive evaluation of protein expression patterns indicative of response to disease, drugs, or the environment. The technology performs up to 100 multiplexed, microsphere-based assays in a single reaction vessel by combining optical classification schemes, biochemical assays, flow cytometry and advanced digital signal processing hardware and software. Details of the procedures have been described elsewhere.[2] Briefly, multiplexing is accomplished by assigning each analyte-specific assay a microsphere set (n=100) encoded with a unique fluorescence signature. Each microsphere set is conjugated with capture antibodies that react with the target protein. After the assay is complete, the microspheres pass single file past two lasers. The red laser (635 nm) excites the encoded dyes to identify the analyte; the green laser

(532 nm) excites a reporter dye to quantify the result.[2-4] Each serum specimen was analyzed for 78 specific serum antigens, 43 autoimmune serologies and 56 infectious disease serologies for a total of 177 analytes in one complex analytical procedure. Serum requirement for these analyses was 100 µl.

We identified 16 analytes measured repeatedly in at least 2 examinations during the course of the first five AFHS physical examinations that are also included in the RBM Human MAP™ for comparison. Eight of the selected analytes had continuous measured levels with normal, laboratory set ranges. These quantitative assays included alpha–1 antitrypsine, C3 complement, creatine kinase, IgA, IgM, PSA, SGOT/AST, and TSH. Results for creatine kinase and SGOT/AST were not directly comparable as RBM MAP panel are non-enzymatic assays. A total PSA was measured in AFHS, free PSA in the RBM panel. Eight other analytes had a positive or negative finding in the AFHS data – these included hepatic panel (HepA Ab, HBs Ag, HBc Ag, HBs Ab, HepC Ab, HepD Ab), mitochondrial antibodies, and thyroidal microsomal antibodies.

Results and Discussion

Two of five selected subjects were Ranch Hand veterans and three were the Comparisons. The dioxin levels for all five randomly selected veterans were below 10 pg/g of lipid. Of the 177 analytes examined, 170 (96%) provided measurable results using RBM's MAP™ technology. Seven analytes provided results not measurable on the standard curve. These analytes are usually present only in pathologic conditions or are considered as potential surrogates of diseases and may be present in very low amounts in serum of relatively healthy subjects (IL-1beta, IL-2, IL-4, IL-6, MMP-9 – inflammation, cancer; glutathion-S-transferase – liver disease, calcitonin – thyroid disease). One hundred forty seven (83%) analytes provided complete results for all analytes in all analyzed samples across all five cycles. Results below standard curves appeared to be subject-related rather than examination cycle/storage time–related, and were not related to dioxin or participation in Ranch Hand or Comparison cohort. We did not observe higher numbers of results below the standard curve for earlier exams than for more recent ones (66 in 1982 samples versus 67 in 1997 samples; results for 23 analytes with at least one result below the standard curve).

Eight analytes with continuous measurements and eight analytes with positive and negative results were identified in AFHS data for comparison with the RBM data. Results for alpha 1 - antitrypsin, IgG, IgM, and TSH are presented in Figure 1a-d; results for the other analytes are described in the text. In Figure 1a, normal ranges for Alpha 1 – antitrypsin were similar for both methods, 0.9-2.2 mg/ml for the AFHS and 1.1-2.7 mg/ml for the RBM MAP. Results for all veterans were within normal ranges. Veteran #5 had consistently lower levels across all measurement cycles but no clear pattern in other veterans' levels was observed. We refer to a clear or similar pattern in results when the same or similar rank and magnitude of measurements emerged in veterans' results from both AFHS and RBM analyses. Alpha 1 – antitrypsin was not measured in 1982 and 1985 in the AFHS but the analyses on stored samples were successful using the RBM panel. Normal ranges for IgA in the AFHS were 0.78-2.86 mg/ml in 1985 and 0.69-3.82 mg/ml for the 1987-1997 examinations (no measurements in 1982). Corresponding ranges in RBM analyses were 0.58 to 5.8 mg/ml. A very similar pattern in the results from both methods is shown in Figure 1b. Additionally, veteran #5 is clearly identified as having higher IgA levels than other veterans but still within normal ranges by both methods. Similar clear patterns emerged for IgM analyses as shown in Figure 1c. Veteran #4 being correctly identified having higher than normal range levels of IgM in the RBM panel corresponding to the AFHS results. In Figure 1d, results from RBM analyses found TSH levels in veteran #5 elevated above normal range in 1982, 1985, 1992, and 1997, similar to AFHS results, and borderline

elevated in 1987. The methods of measurements and normal ranges changed in AFHS over the first three examinations but overall patterns for TSH were similar in both methods.

Normal ranges for C3 complement were 0.85-1.93 mg/ml in AFHS and 0.89-2.5 mg/ml in RBM analyses. All results fell within the normal ranges for both sets of measurements without a clear distinguishing pattern in veterans' levels. A total PSA was measured in 1992 and 1997 in the AFHS and free PSA in the RBM panel (for all examinations), but the patterns in 1992 and 1997 were similar for both measurements. For SGOT/AST and creatine kinase we would not expect the results to agree as an enzymatic method was used to measure these enzymes in the AFHS data. Amount or weight measured immunologically was ascertained by the RBM panel but the analyses were performed on all samples successfully.

None of the five selected veterans had a presence of mitochondrial or thyroid microsomal antibodies detected in 1992 or 1997 in the AFHS data. RBM technology assays were able to run analyses in samples from those exam cycles and also for 1982-1987 examinations and similarly found no positive reaction. A presence of HepA antibodies was detected in two veterans at 1987 and 1992 examinations, and in three veterans at the 1997 examination in the AFHS data. RBM analyses did not detect the antibodies in the same veterans. The discrepancy in the results may have been due to a different HepA serotype used to prepare the MAP HepA assay or other methodologic issues. There was one veteran with positive HBs antibodies detected in 1982 and through following examinations; this veteran was also positive for HBc Ab. The RBM analysis correctly identified the veteran and confirmed these findings. No positive results were found for HepC Ab or HepD Ab by either assay.

In conclusion, 96% of all 177 analytes provided detectable results using high-density immunoassays performed by RBM's Human MAP^{TM} technology using only 100 µl of sera. This technology appears be a useful instrument in analysis of frozen serum samples that were stored for 9 to 24 years, especially in cases where a limited amount of sera is available and analyses of a large number of analytes are required. These results suggest that the stored AFHS samples represent a great source of phenotypic data that is well preserved and could be successfully used for future examinations of protein pattern expressions related to environmental, disease or drug exposures.

Acknowledgements
We would like to thank Rules-Based Medicine for timely analysis of serum samples and their assistance in preparation of this manuscript. We also thank SAIC for facilitating the contract arrangements.

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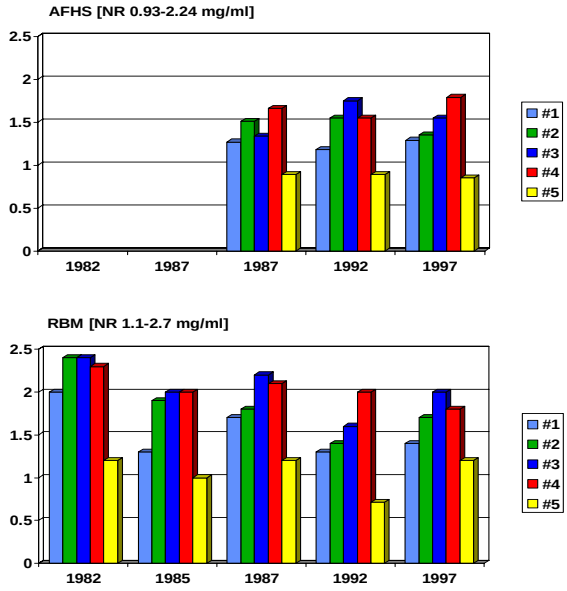
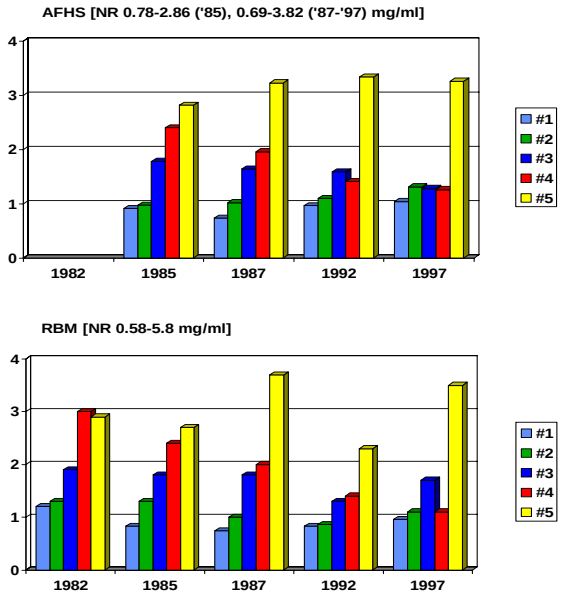
Figure 1. Comparison of selected biochemical parameters from AFHS and RBM analyses in 25 stored frozen serum samples (1982-1997).

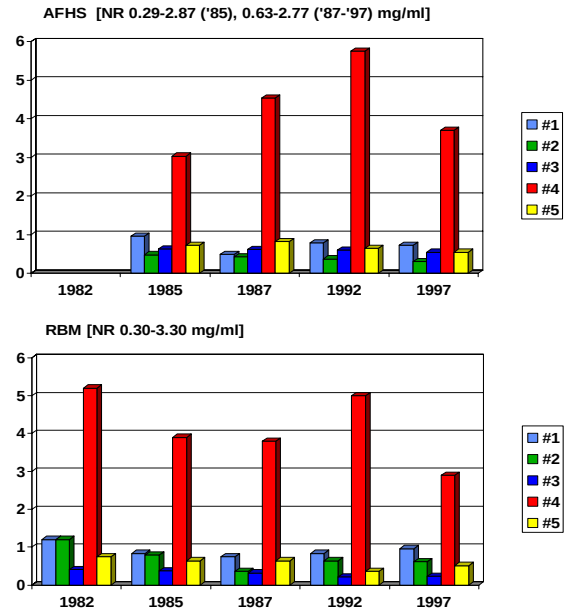
a) Alpha-1 antitrypsin



b) Immunoglobulin A (IgA)



c) Immunoglobulin M (IgM)



d) Thyroid Stimulating Hormone (TSH)