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Honors Scholar Thesis

University of Connecticut *Year 2008*

Biological Signatures of Vaccine Responses

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ABSTRACT

The set of host- and pathogen-specific molecular features of a disease comprise its "signature". We hypothesize that biological signatures enables distinctions between vaccinated vs. infected individuals. In our research, using porcine samples, protocols were developed that could also be used to identify biological signatures of human disease. Different classes of molecular features will be tested during this project, including indicators of basic immune capacity, which are being studied at this instance.

These indicators of basic immune response such as porcine cytokines and antibodies were validated using Enzyme-linked immunosorbent assay (ELISA). This is an established method that detects antigens by their interaction with a specific antibody coupled to a polystyrene substrate. Serum from naïve and vaccinated pigs was tested for the presence of cytokines. We were able to differentiate the presence of porcine IL-6 in normal porcine serum with or without added porcine IL-6 by ELISA. In addition, four different cytokines were spotted on a grating-coupled surface plasmon resonance imaging system (GCSPRI) chip and antibody specific for IL-8 was run over the chip. Only the presence of IL-8 was detected; therefore, there was no cross-reactivity in this combination of antigens and antibodies. This system uses a multiplexed sensor chip to identify components of a sample run over it. The detection is accomplished by the change in refractive index caused by the interaction between the antibody spotted on the sensor chip and the antigen present in the sample.

As the multiplexed GCSPRI is developed, we will need to optimize both sensitivity and specificity, minimizing the potential for cross-reactivity between individual analytes. The next step in this project is to increase the sensitivity of detection of the analytes. Currently, we are using two different antibodies (that recognize a different part of the

antigen) to amplify the signal emitted by the interaction of antibody with its cognate antigen.

The development of this sensor chip would not only allow to detect FMD virus, but also to differentiate between infected and vaccinated individuals, on location. Furthermore, the diagnosis of other diseases could be done with increased accuracy, and in less time due to the microarray approach.

ABBREVIATIONS

Enzyme-linked immunosorbent assay, ELISA

Grating-coupled surface plasmon resonance imaging, GCSPRI

Surface plasmon resonance, SPR

Region of interest, ROI

Bovine serum albumin, BSA

Foot-and-mouth disease, FMD

Tumor necrosis factor alpha, TNFα

Interleukin 4, IL-4

Interferon gamma, INFγ

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INTRODUCTION

Currently, the diagnosis of infectious diseases is often limited to the identification of the causative pathogen. We hypothesize that the molecular characteristics of the host response produced as a result of an infection will provide an improved tool for its diagnosis, and will also lead to better therapeutic managements of these diseases. The set of host- and pathogen-specific molecular features of a disease comprise its "signature". For example, a breast cancer signature has recently been developed. The results of the study show that the signature was more accurate in selecting the patients assigned to good versus poor prognosis groups. For instance, while using the traditional methods based on histological as well as clinical characteristics, only between 7 and 15% of patients were assigned to the good prognosis group; 40% of them were assigned to the same group according to the signature approach. Follow up showed that the patients in the good prognosis group, according to the signature, were less likely to develop metastasis [1].

We are employing Grating-coupled Surface Plasmon Resonance Imaging (GCSPRI), an instrument that allows for the simultaneous detection of different analytes from the same sample (including proteins, DNA, cells, bacteria and viruses) using the same chip. The GCSPRI instrument uses changes in the refractive index caused by analyte capture by the sensor chip to identify the presence of the analyte.

Traditional Surface Plasmon resonance (SPR) measures the interaction of an antibody-antigen pair by detecting changes in the refractive index of an aqueous medium near a metal surface [2]. In order for the resonance (plasmon) to occur certain conditions such as wavelength and angle of incidence have to be met. The Kretschmann configuration (Figure 1) uses a prism, which has a high index of refraction to produce the necessary conditions for the surface plasmon to occur. The GCSPRI technology uses a

sensor chip that contains a grating on the surface that allows for the deflection of light at different angles (Figure 2). When the velocity and momentum of some of these waves coincide with the ones of the plasmon, electrons on the surface of the chip are coupled forming a surface plasmon [2]. Therefore, there is a need to use a prism or two different mediums such as liquid and metal surface to create the resonance and detect the interaction.

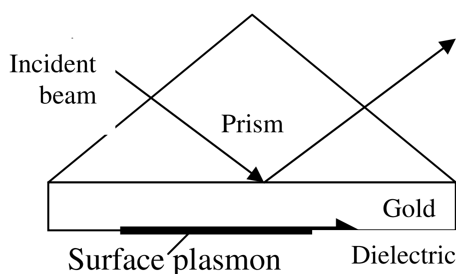

Figure 1. Kretschmann prism configuration [3]

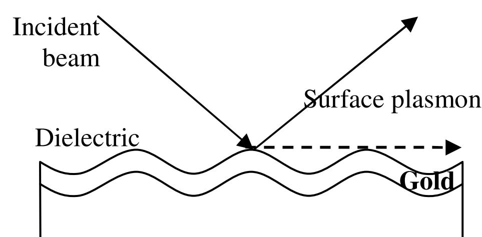

Figure 2. Grating-coupled chip configuration [3]

Our work is concerned with the identification of a biological signature that will enable the distinction between vaccinated vs. infected hosts. This signature comprises pathogen-and host-specific molecular features that are specific for Foot-and-mouth disease. Using only one marker, such as the concentration of antibodies against Foot and Mouth disease virus present in serum, would not be sufficient to make this distinction. In order for the vaccine to work, the antigen introduced to the body would have to be a part or an inactivated form of the virus. Therefore, the distinction between the antibodies against a live or inactivated virus would be very difficult to make since both would have the same components. Even if this distinction could be made, the concentration of antibodies or the virus could vary among individuals. Therefore, besides looking at one or

two features, the animal's immune response would be tested in order to make that distinction. This signature approach would make a more accurate detection of infection because there would be dozens of features tested and evaluated statistically.

The research is being done using porcine samples, but the same protocols could be used to develop biological signatures of human disease. There are four classes of molecular features to be tested: (1) indicators of basic immune capacity (antibody levels, levels of cytokines ("immune hormones"), and numbers of immune cell subpopulations), (2) indicators of specific immune response (antibodies specific for the different viruses or bacteria tested), (3) indicators of tissue damage or cellular stress (heat shock proteins and metallothionein), and (4) indicators of autoimmunity (presence of antibodies against host cellular components).

Our project looks for differences in molecular features of cattle infected with or vaccinated against Foot-and-mouth disease virus. FMD is caused by a single-stranded, plus-sense RNA virus. It has several serotypes that have been found in different parts of the world, and they include A, O, C, Asia1, and South African Territories 1, 2, and 3 [4]. This disease does not have a high mortality rate, however, it severely decreases the animal's productivity, and since it spreads fairly easily, all animals part of the herd need to be killed. Furthermore, infected animals cannot be sold internationally, and vaccination limits the market for cattle due to the difficult differentiation between infected and immunized groups. Also, if the inactivated virus used in vaccines is not fully inactivated it could cause the transformation of the virus to the wild type causing infections [5]. Therefore, the financial burden for the owners of infected herds is very high. The last major out-break of FMD happened in Great Britain in 2001, and it caused the loss of thousands of cattle world-wide along with its financial consequences. There is a need for

developing an assay that could rapidly and accurately detect the presence of this virus. This way the infected animals could be isolated preventing the spread of the disease. Since some of the FMD symptoms are similar to other diseases such as vesicular stomatitis, it is necessary to have the ability to test specifically for the presence of FMDV. One of the challenges of this project is that no laboratory in the continental United States is allowed to have the virus. We are currently working with pigs vaccinated with a synthetic protein of the virus.

Designing an assay to test a specific signature has some added challenges. For instance, all the animals in the herd have different genetic characteristics, and will react to an infection in a different way. Even if they are infected by the same virus at the same time, their immune systems might react differently depending on each individual's health. Polymorphisms in cytokine genes might cause individuals to have different concentrations of cytokines at any given time. Also, mutations in the genes that code for the receptors of B and T lymphocytes might cause them react differently when a pathogen infects the body. Therefore, the parameters of the signature have to be carefully studied to minimize the errors.

We are searching for statistically significant differences in the serum levels of analytes that would allow us to differentiate between infected versus vaccinated individuals. Table 1 shows some examples of how cytokines vary according to the individual's circumstances. Our initial studies addressed how specific the interactions between antigen and antibody are, and what are the limits in the detection of each analyte. One of the pitfalls is that porcine antibodies have not been previously adsorbed; therefore, they might interact non-specifically with other serum components. Also, when running the different antibodies sequentially in GCSPRI, some might interact with more than one

analyte. As a consequence, it will be difficult to determine the concentration of each antigen in serum once all the analytes are spotted on the chip. In order to decrease the cross-reactivity issues, we will use antibody pairs. In this way each component of the pair will recognize a different part (epitope) of the same analyte (Figure 3). The first antibody will be adsorbed to the matrix of the plate. Then, the antigen (in serum) would be allowed to bind. The second antibody, which would be coupled to an enzyme, would bind to a different part of the antigen. Each step will be followed by washing (as explained in the ELISA protocol) to eliminate the unbound molecules. Then, the substrate for the coupled enzyme would be added; and this reaction would produce color. The change in color would be representative of the amount of antigen captured by the first antibody and bound to the second one.

Table 1. Concentration of IL-2, IL-6, IL-8, and TNFa in the serum of smokers and non-smokers suffering from schizophrenia (ng/ml) [6]. This table shows an example of a signature (neither schizophrenia nor smoking are related to our project).

	Smokers	Non-smokers
IL-2	4.1±2.3	7.6±7.4
IL-6	0.34±0.11	0.41±0.10
IL-8	0.98±0.30	1.1±0.28
TNFa	10.1±1.8	10.7±2.7

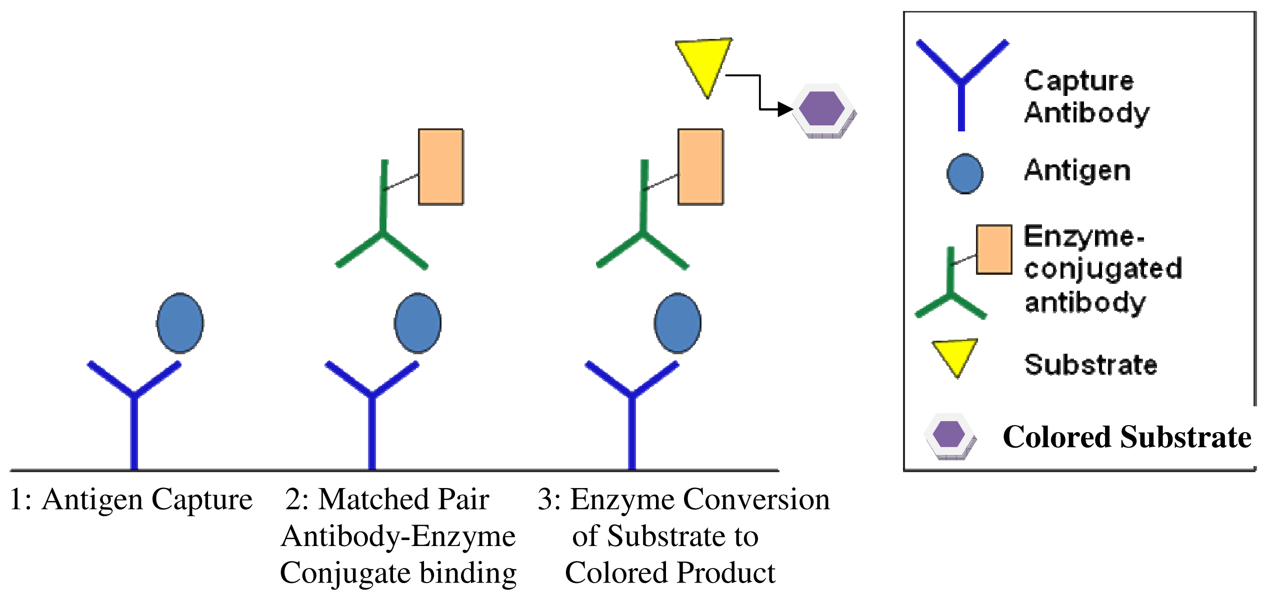

Figure 3. Diagram of sandwich ELISA

MATERIALS AND METHODS

Reagents

Recombinant porcine TNFα, IL-4, INFγ, IL-10, IL-2, IL-1β, IL-6, IL-8 mouse anti-porcine IL-4, mouse anti-porcine INFγ, were purchased from Cell Sciences (Canton, MA). Mouse anti porcine TNFα, native porcine IgM, mouse anti-porcine IgM were purchased from AbD Serotec (Raleigh, NC). Mouse anti-porcine IL-10, mouse anti-porcine IL-2 were purchased from R&D Systems (Minneapolis, MN). Mouse anti-porcine IL-1β, mouse anti-porcine IL-6, mouse anti-porcine IL-8, recombinant porcine IL-12 p70, mouse anti-porcine IL-12 p70 were purchased from Pierce Endogen (Rockford, IL). Porcine IgG was obtained from Bethyl Laboratories, Inc (Montgomery, TX). Rabbit anti-mouse IgG-AP conjugated was purchased from Fisher Scientific (Pittsburg, PA).

Enzyme linked immunosorbent assay (ELISA).

96-well 2 HB Immulon plates (Thermo Labsystems, Franklin, MA) were coated with antigens or antibodies depending on the assay. After the plates were incubated for 1 hour at 37ºC, they were washed three times with PBST (137 mM NaCl, 2.6 mM KCL,1.6mM Kh2PO4, 1mM Na2HPO4,0.05% Tween 20 and 0.2% NaN3 , pH 7.2) in an automated plate washer (Biotek, Burlington, VT). Then, each well was blocked with 200 µl of 2% BSA in PBST for 1 hour at 37ºC. The plates were washed again, and coated with 100 µl of the corresponding antigen or antibody. The plates were incubated for 1 hour at 37ºC, and washed. Then, 100µl of a 1:2000 dilution of rabbit-anti-mouse-IgG coupled with Alkaline Phosphatase was added to each well. Again the plates were incubated for 1 hour at 37ºC, and washed for the last time. Finally, each well was coated with 100 µl of p-Nitrophenyl Phosphate, Disodium salt (PNPP, Sigma-Aldrich, St Louis, MO) in substrate

buffer (1mg/ml). The change in color was measured in a plate reader (Molecular Devices, Menlo Park, CA) at 405 nm for 10 minutes.

Microarray preparation

The GCSPR chips were washed with 70% ethanol and rinsed with distilled water to remove any possible contamination. The chips were air-dried and printed using the MicroCASTer Pin System (Schleicher & Schuell Inc.). The different porcine antigens, and control molecules (BSA) were each spotted on the gold surface of the chip (50 nL/spot) at different concentrations. The chips were then incubated for 1 hour in a humidified, 37°C incubator to allow for protein adsorption to the gold surface. After the chips were removed from the incubator, they were placed in a dessicator at 4°C overnight, before use in the GCSPR instrument.

GCSPR assay

Once installed in the GCSPR instrument's flow cell, the chips were blocked with 2% BSA in PBS (3x5ml flushes followed by 5 min incubations). Then they were washed with PBS (20 min at 250 µl/min) and angle scans were done in order to measure the initial baseline GCSPR angle. Sequential angle scans that measure GCSPR angle shifts were performed throughout the experiment. The different analytes were passed across the sensor chip surface for 30 min at 100 µl/min, after a PBS run of 10 min at 100 µl/min. The same PBS run was done in between the analyte runs.

RESULTS

The available porcine cytokines were validated using ELISA. The detection of each cytokine, which was plated at a concentration of 25ug/ml, ranges from 4.48 to 259.33 mDeg/min. This variation could be due to the different sizes of the cytokines, and the specificity of the antibodies used. Most of the validation experiments were duplicated with comparable results.

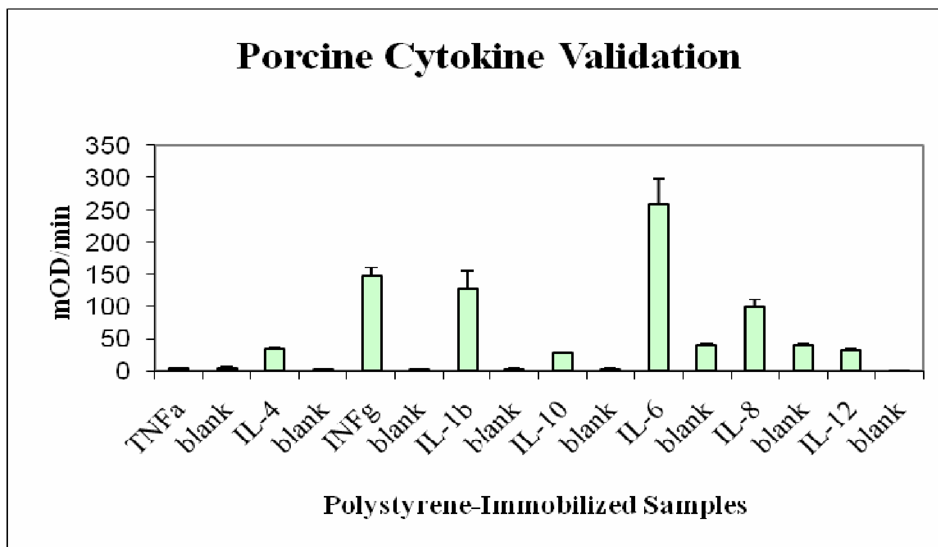

Figure 4. Porcine cytokine validation by ELISA. Procedure: PBS was added to the samples to achieve a concentration of 25ug/ml. Then, they were non-specifically immobilized to the polystyrene microtiter plate by direct adsorption. After blocking with 2% BSA, monoclonal mouse anti-porcine antibodies (10ug/mL) were incubated in each well, followed by rabbit anti-mouse IgG conjugated with alkaline phosphatase (AP) at a concentration of 0.5ug/mL. PNPP was used as a substrate for the AP. Each step was followed by a 1-hour incubation period at 37 degrees and automated washing in a microplate washer. This is a compiled graph from different experiments. As soon as each cytokine was bought, it was validated and the results are shown in the graph.

Porcine IL-6 gave the highest level of detection in the validation experiment; therefore, it was the first one to be tested. The cytokine was added to normal porcine serum (not immunized with synthetic FMD protein) to be detected by mouse anti-porcine IL-6 added afterwards. The unchallenged serum without added porcine IL-6 showed detection in the same range as the porcine IL-6 alone. This shows that there was IL-6 present on the serum or that the antibodies used reacted with some other serum components. The unchallenged serum with added IL-6 showed a high level of detection. This result further validate that the mouse anti-porcine IL-6 was able to detect the IL-6 added.

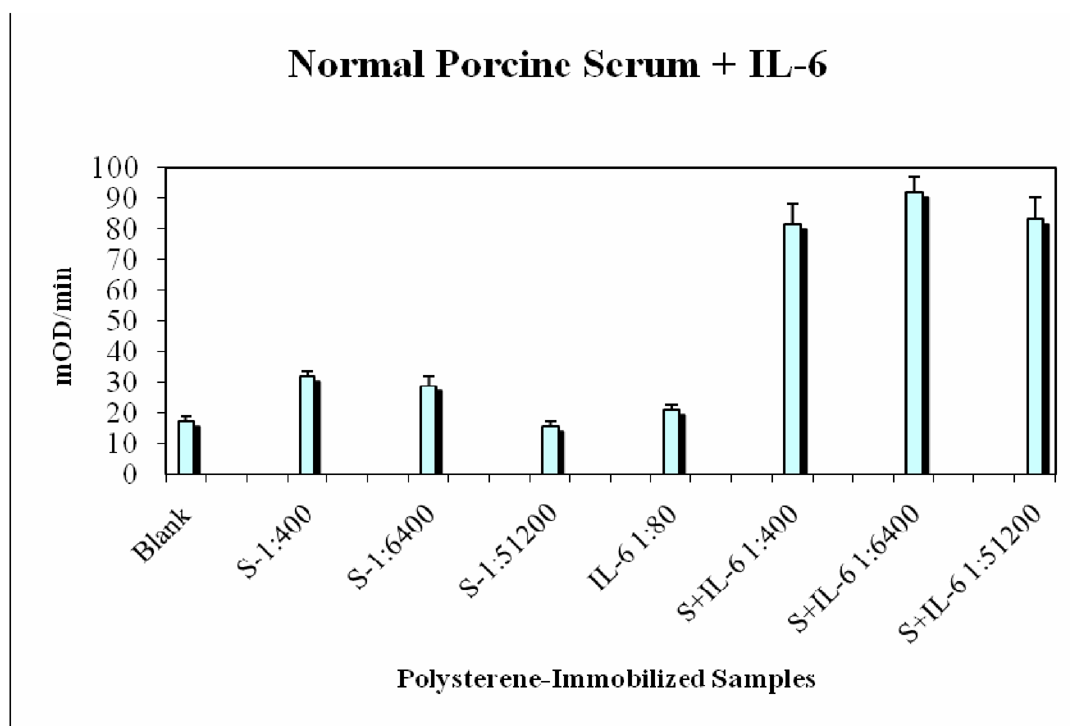

Figure 5. Binding of mouse anti-porcine IL-6 to normal porcine serum (S) with or without added porcine IL-6 measured by ELISA. Procedure: PBS was added to samples, which were then non-specifically immobilized to the polystyrene microtiter plate by direct adsorption. After blocking with 2% BSA, monoclonal mouse anti-porcine IL-6 (10ug/mL) was incubated in each well, followed by rabbit anti-mouse IgG conjugated with alkaline

phosphatase (AP) at a concentration of 0.5ug/mL. PNPP was used as a substrate for the AP. Each step was followed by a 1-hour incubation period at 37 degrees and automated washing in a microplate washer. Comparisons were made between wells to which no IL-6 was added (blank), unsupplemented serum at a range of dilutions (S), IL-6 alone at a dilution of 1:80 (1.25ug/ml), and serum at these dilutions, to which IL-6 had been added at a concentration equivalent to that in the IL-6 alone wells.

To test the specificity of the antibodies, four cytokines were spotted on a sensor chip for GSPRI. Mouse anti-porcine IL-8 was passed across the sensor chip surface. The data shows the highly specific interaction between the porcine IL-8 antigen and antibody pair. There was some non-specific interaction of the antibody and the BSA.

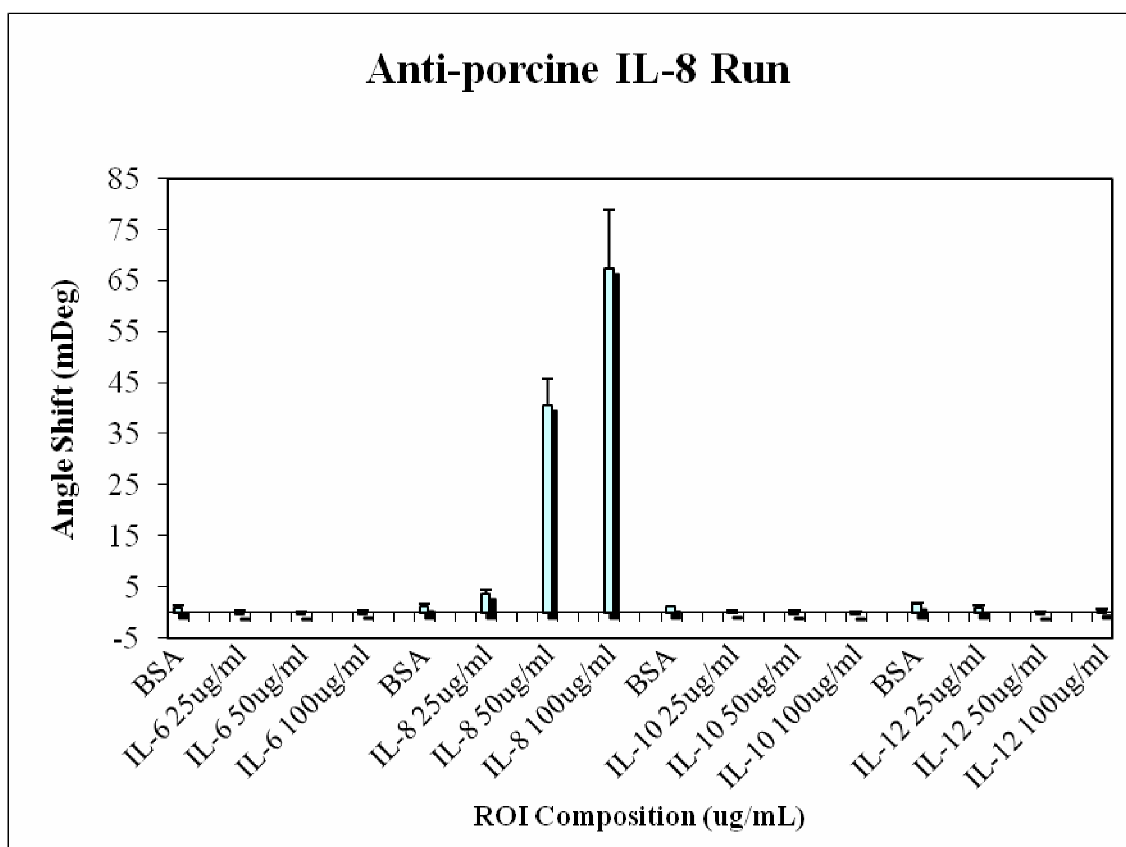

Figure 6. Binding of mouse anti-porcine IL-8 to porcine IL-8 that was immobilized on a gold chip measured by GCSPR. Procedure: 0.005% BSA in PBS was added to porcine IL-6, IL-8, IL-10, and IL-12, which were then non-specifically immobilized to the gold chip by direct adsorption. After blocking with 2% BSA, a solution containing the anti-IL-8 antibody was run at 100uL/min for 30minutes. A PBS flush followed the sample run. The anti-IL-8 was at a concentration of 20ug/mL. The positive angle shifts show that there was specific binding of mouse anti-porcine IL-8.

In order to further test the specificity of the antibodies, another experiment was set to test the mouse anti-porcine INFg. Some non-specific substances such as albumin, BSA, and fish gel along with porcine INFg were spotted on a sensor chip. Mouse anti-porcine INFg was run over the chip. The data shows a high level of specificity between the porcine INFg and its antibody. However, at the lowest INFg concentration (25ug/ml) the binding is comparable to the fish gel at 12.5ug/ml.

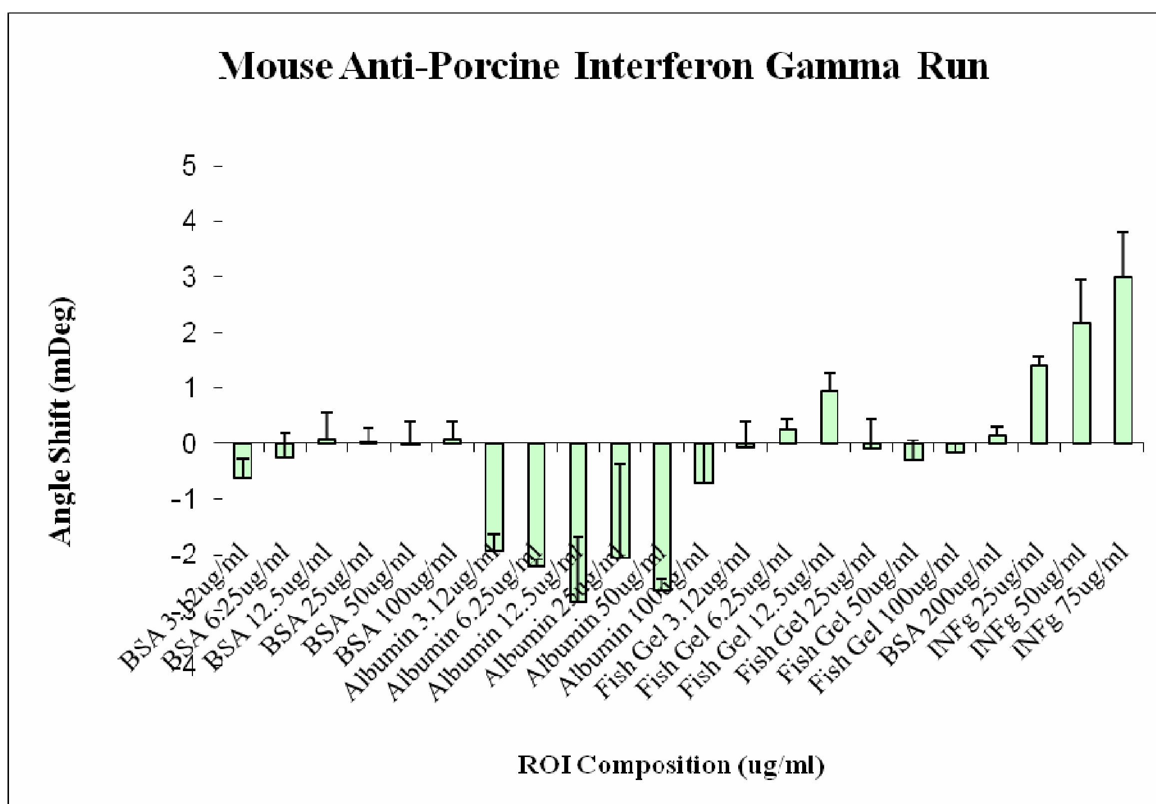

Figure 7. Binding of mouse anti-porcine INFg to porcine INFg, BSA, albumin, and fish gel, immobilized on a gold chip measured by GCSPR. Procedure: PBS was added to porcine INFg, BSA, albumin, and fish gel, which were then non-specifically immobilized to the gold chip by direct adsorption. After blocking with 2% BSA, a solution containing the anti-INFg antibody was run at 100uL/min for 30minutes. A PBS flush followed the sample run. The positive angle shifts show that there was specific binding of mouse anti-porcine INFg to its cognate antigen with some cross-reactivity to fish gel.

DISCUSSION

This project was developed under the hypothesis that a biological signature would allow distinction between vaccinated versus infected hosts with FMD. Even though that could not yet be determined, some advancements have been accomplished. One of the limitations encountered was the small amounts of critical regents that were available. This restricted the experiments that could be done. Cytokines are not usually used for coating the plate (as was done in the first ELISA experiments). The standard procedure for detecting cytokines in serum is by using a sandwich arrangement with an antibody pair that recognizes different epitopes on the antigen. This arrangement minimizes the amount of the cytokine used to obtain detections in the range that the cytokines are present in serum.

Another difficulty encountered was that the porcine antibodies were not sufficiently specific. There was some cross reactivity between the secondary antibodies used to detect the mouse anti-porcine cytokines, and other experiment components such as BSA (data not shown). When testing for the presence of porcine IL-2 in serum, the concentration of IL-2 encountered did not decrease proportionally with decreasing serum concentrations. One of the possible explanations is that the goat anti-mouse IgG was detecting some serum component other than the primary antibody. As a consequence, a concentration curve could not be developed.

In established procedures, the laboratories that produce the reagents test them for cross-reactivity with the species used more frequently. However, since this is a novel experiment, there are no standards to be followed. Also, the possibility that when testing porcine or bovine sera on location there could be antibodies against other animals has to be contemplated when designing the sensor chip. This problem did not happen in GCSPR

experiments since the secondary antibody is not used. Therefore, the cross-reactivity is highly dependent on the type of assay used in the experiments.

The first component of this project was to validate the reagents using ELISA (Figure 4). Then, serum from naïve and vaccinated pigs was tested for the presence of cytokines. The presence of porcine IL-6 was detected in normal porcine serum with or without added porcine IL-6 by ELISA (Figure 5). Furthermore, the presence of IL-8 was specifically detected when other cytokines were also spotted on the same sensor chip used in a grating-coupled surface plasmon resonance imaging system (Figure 6).

References with respect to the concentration of cytokines in porcine serum has being difficult to find. However, according to the values presented in Table 1, we are still far from the desired detection range (assuming similar concentration of cytokines in pig). Our smallest detection is in the hundred nanogram per milliliter range. Optimizing both sensitivity and specificity, and minimizing the potential for cross-reactivity between individual analytes can be accomplished by using the sandwich arrangement explained above.

The road for the completion of this project lies ahead. However, the results obtained so far are very promising. The maximization of the detection levels as well as the minimization of cross-reactivity while spotting more and more components of the signature on the sensor chip are the next steps to follow.

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