Use of Random Peptide Reactivities to Analyze Host Immune Responses

of

African Swine Fever Virus Infection and Immunization

by

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ABSTRACT

African Swine Fever (ASF), endemic in many African countries, is now spreading to other continents. Though ASF is capable of incurring serious economic losses in affected countries, no vaccine exists to provide immunity to animals. Disease control relies largely on rapid diagnosis and the implementation of movement restrictions and strict eradication programs. Developing a scalable, accurate and low cost diagnostic for ASF will be of great help for the current situation. CIM's 10K random peptide microarray is a new high-throughput platform that allows systematic investigations of immune responses associated with disease and shows promise as a diagnostic tool. In this study, this new technology was applied to characterize the immune responses of ASF virus (ASFV) infections and immunizations. Six sets of sera from ASFV antigen immunized pigs, 6 sera from infected pigs and 20 sera samples from unexposed pigs were tested and analyzed statistically. Results show that both ASFV antigen immunized pigs and ASFV viral infected pigs can be distinguished from unexposed pigs. Since it appears that immune responses to other viral infections are also distinguishable on this platform, it holds the potential of being useful in developing a new ASF diagnostic. The ability of this platform to identify specific ASFV antibody epitopes was also explored. A subtle motif was found to be shared among a set of peptides displaying the highest reactivity for an antigen specific antibody.

However, this motif does not seem to match with any antibody epitopes predicted by a linear antibody epitope prediction.

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Chapter 1

INTRODUCTION

Overview

African swine fever (ASF) is a hemorrhagic disease that is widespread in Africa and continues to spread to other continents. It is considered as List A disease by the Office International des Epizootics (OIE). Due to its high mortality rates and its wide host range, it can cause major economic losses in those affected countries (Costard, Wieland et al. 2009). ASF is caused by ASF virus (ASFV), a large double-stranded DNA virus that was classified as the only member of the Asfarviridae family. ASFV can infect both wild and domestic porcine species. There is no effective vaccine available for ASFV. The control and eradication strategies mainly rely on rapid and effective laboratory diagnosis, the enforcement of strict sanitary regulations, and slaughter. Although recently developed techniques such as real-time PCR tests have proved to be useful in identifying ASFV infection, detection of ASFV-specific antibodies remains the major method of diagnosis. A antibody profiling assay based on our center's 10K random peptide microarray has been successfully used to investigate the disease-associated changes for many diseases (Boltz, Gonzalez-Moa et al. 2009; Legutki, Magee et al. 2010; Halperin, Stafford et al. 2011). In this study, we took advantage of this new technology to analyze host immune responses to ASFV relative to viral infection and antigen immunization and explored its potential for ASF diagnosis.

Statement of Problem

Similar to other viral diseases, ASF diagnosis is typically based on the detection of infectious virus, viral antigens, viral DNA or viral specific antibodies. Serum antibodies are not only used as indexes of ASFV infections, but also can be used to study sub-acute and chronic forms of ASF (Perez-Filgueira, Gonzalez-Camacho et al. 2006). Therefore, although many laboratory tests for ASF are available, the serological studies are the most important. The Enzyme-Linked Immunosorbent Assay (ELISA) test is currently the most useful serological method for large scale diagnosis (Gonzague, Roger et al. 2001). Viral antigens used in the ELISA tests are either derive from live virus or recombinantly produced. Viral antigens derive from virus involve manipulation of the infectious agent thus have safety concerns and the production of the recombinant viral proteins is not cost efficient (Perez-Filgueira, Gonzalez-Camacho et al. 2006). Thus, a cheap and scalable serological diagnosis is desired.

Chapter 2

Background and Significance

ASFV

ASF is a highly contagious and fatal viral disease of pigs caused by ASFV, a complex virus that belong to the Asfarviridae family. ASFV has a double stranded linear DNA genome that varies from 170 Kb to 190 Kb and encodes between 160 to175 genes, depending on the isolate (Costard, Wieland et al. 2009). ASF was first described in Kenya in 1921. Since then, it has been acknowledged as endemic disease in many African countries. The first outbreak of ASF outside Africa occurred in Lisbon (Portugal) in 1957. The most recent outbreak of ASF outside Africa started at the beginning of 2007 in Georgia, and has since spread to the countries of Armenia, Azerbaijan, Iran and Russia (Rowlands 2008). ASF appears mostly as an acute disease in Africa, but mainly subacute or chronic forms are found outside of Africa. Clinically, ASF can present a range of clinical signs. Some of them are similar to other diseases of pigs. The immune mechanisms that related to protection against ASF are not thoroughly understood. Although many efforts have been made to develop an effective vaccine, there has been no success to date. Effective treatments or cures for ASF are also not available at this point.

Although the United States was listed as one of the top world pork import and export countries, efficient surveillance and strict control and prevention policies can help us from ASF outbreaks. However, most of

African countries, including many countries of South America are less developed countries can be easily exposed to the consequences of an introduction of ASFV due to their poor surveillance systems and ineffective control. Also, extreme climatic events like earthquake and other disasters can make these less developed countries even more vulnerable to the spread of ASFV. More importantly, many of families from those countries are low-income rural families. The infected pigs could be the only livestock they owned. Slaughtering their only livestock could be a huge loss to them. An accurate detection method would largely improve the chances of the disease control and prevent the tragedy from occurring to those poor families. Such a detection method must also be cost efficient in order to make large scale assessments. Apart from economic consideration, an ideal detection of ASFV should also be rapid and accurate, which would allow a timely diagnosis and ensure the management of the risk of introduction and spread of the disease. However, there are many difficulties involved in finding such detection methods. Firstly, ASFV have many isolations and it present a lot variations in pathogenesis level which make an accurate diagnosis to be very difficult. Also, the similarities of the clinical symptoms between ASF and other hemorrhagic diseases, such as classical swine fever (CSF), erysipelas, and septicemic salmonellosis. It also make the diagnosis more sophisticated (Gallardo, Blanco et al. 2006).

ASFV diagnosis

Currently there are many different laboratory tests have been used for the identification of ASFV. Like other viral infections, these diagnoses of ASF are based on the demonstration of the infectious virus, viral antigens, viral DNA or specific antibodies(Barbara E. Straw 2006). The major tests are listed as follow:

(1) Hemadsorption test. ASFV can be isolated by inoculating blood or tissue samples from suspect pig in to leukocyte or bone marrow cultures. Most isolates of ASFV can induce hemadsorption of pig erythrocytes to the surface of the infected cells. This test is very sensitive and used to confirm new outbreaks. However, the non-hemadsorbing isolates can be missed by this test(Bustos, Nogal et al. 2002).

(2) Direct immunofluorescence. It is based on detecting viral antigen in impression smears or frozen tissues sections which react with conjugated ASFV antibodies. It is a fast test for acute form of ASF, but has low diagnostic sensitivity for chronic forms of ASF. The decreased sensitivity may relate to the formation of antigen-antibody complexes in tissue could block the interaction between the ASFV antigen and *in vitro*-added antibody conjugate(Hess 1981).

(3) Polymerase Chain Reaction (PCR) assay. PCR assay can be used to detect a highly conserved region of the viral genome. It has been used to identify all the ASFV isolates (Aguero, Fernandez et al. 2003). PCR assay is very useful for detecting acute viral isolates which can kill pigs

before the antibody responses is mounted. It is also very helpful in putrefied samples that cannot be used for other diagnostic test. Real time PCR tests that was recently developed have also been proved to be a useful tool for identifying ASFV in infected animals (Zsak, Borca et al. 2005; Ronish, Hakhverdyan et al. 2011). However, these tests require sophisticated equipment and expensive ingredients that not readily available in endemic areas.

(4) Serologic tests are based on the detection of ASF antibodies that can persist for long periods after infection. It is widely used in endemic regions(Gallardo, Blanco et al. 2006). Although many serologic tests have been developed, ELISA is the most widely used tool for large scale diagnosis. It has also been standardized for routine used. However, the scopes of information provided by the ELISA assay readouts are limited and the antigens used these assays are not cheap to produce. Given the shortage of ELISA tests, a high throughput microarray seems to be a good solution.

CIM 10K random peptide microarray

The CIM 10K random peptide microarray consists of approximately 10,000 random peptides. These 20-mer random sequence peptides were predicted using custom software that generates random natural amino acids sequences. Nineteen amino acids (except cysteine) were available for selection in each of the amino-terminal seventeen positions, and then Glycine-Serine-Cysteine were uniformly added to the carboxy-terminus as

linkers to keep the other 17 functional residues raised from the glass surface(Legutki, Magee et al. 2010). This high throughput screening platform has been successfully used in many different applications: identifying surface-immobilized peptides which specifically bind bacterial lipopolysaccharides (Boltz, Gonzalez-Moa et al. 2009; Morales Betanzos, Gonzalez-Moa et al. 2009), guiding production of synthetic antibodies(Williams, Diehnelt et al. 2009), characterizing humoral response to influenza infections and vaccination, and evaluate Alzheimer's disease(Legutki, Magee et al. 2010; Lucas Restrepo and Johnston 2011).

Chapter 3

RESEARCH HYPOTHESIS

Although the immune mechanisms that protect against ASF are not well understood, we do know that ASFV can elicit high levels of specific antibodies during the infection. ASFV IgM can be detected four days after infection and ASFV IgG can be detected in the blood six to eight days after infection(Sánchez-Vizcaíno 1999). Several widely used diagnostic methods that detect antibody responses such as ELISA and immunoblotting assay are all based on such knowledge. The CIM 10K random peptide microarray consists of 10K random 20 mer peptides that are covalently attached at their C-terminus to the glass surface. It has been proved to be a very successful platform for detecting many antibody reactivates (Boltz, Gonzalez-Moa et al. 2009; Morales Betanzos, Gonzalez-Moa et al. 2009; Legutki, Magee et al. 2010). We believe such a platform can be used to compare and contrast the antibody reactivates of ASFV antigen immunized pigs and ASFV infected pigs. Our recent ELISA data that demonstrated that our immunized and non-immunized sera carried differences in antibody levels to specific antigens, suggest that these sera are appropriate for deeper analysis using our 10K random peptide microarray. We hypothesize that our microarray based system can distinguish between viral infected pigs from uninfected pigs. Furthermore, we hypothesize that this immune assay platform can be used to distinguish the antibody responses of infected vs. subunit vaccinated pigs.

We also want to test if our system can differentiate ASFV infected pigs from pigs infected by other viruses, which has the potential to be very practical in the diagnosis for ASF in field settings. Moreover, we want to test the sensitivity and the specificity of our random peptide microarray by comparing the immune profiles of pigs that were immunized with a set of 42 different ASFV antigens. The 10K random peptide microarray platform may be able to identify random peptides for use in development of a simple ASFV diagnostic.

Chapter 4

MATERIALS AND METHODOLOGY

Research Design

Overview

All 48 sera samples were used to probe the CIM 10K random peptide microarrays. The scanned images of the slides were then aligned by using GenePix Pro 6.0. The median intensities of the peptide signals (expression level) were measured and averaged across the replicates. Then the signal intensities of all the peptides were then loaded into Genespring GX 7.3. To explore the diagnostic potential of our random peptide microarray, seven different comparisons of different groups of sera were evaluated using one-way analysis of variance (ANOVA) and principle component analysis (PCA). To determine the antibody binding pattern of different group of sera, the most preventative peptides that were selected by ANOVA were used to create the heat map and reveal the PCA plot.

To identify the peptides that can bind to ant-VP30 antibody, the anti-VP30 antibody depletion test was carried out. VP30 protein was synthesized *in vitro* by using VP30 linear expression elements (LEE) as the template, then incubated with ASFV sera to deplete anti-VP30 antibody. After depletion test, the anti-VP30 antibody was separated from VP30 protein by glycin-HCl elution. Both depleted sera and purified anti-VP30 antibody were collected to probe the 10K random peptide

microarray. The peptides that bind to anti-VP30 antibody were then compared with the VP30 protein sequence by a motif finding program GLAM2SCAN, in order to identify the sequence similarities. Also, these peptides were compared with the antibody binding epitopes that predicted by Bepipred Linear Epitope Prediction.

Serum Sources

Immunized British sera. Four groups of the immunized British sera samples were from the ASF vaccine discovery project. The objective of this project is to develop a safe and effective component vaccine for ASF by vaccinating the pigs with different groups of viral proteins. Such a strategy allows us to screen for potentially protective antigens that are not normally exposed to or recognized by the immune system. The immunizations were conducted with a gene-prime and vaccinia-boost regimen. Twenty pigs were split into four groups, five pigs each group. Pigs in group 1 were genetically immunized with two known ASFV immunogens, VP30 and VP72, along with a pool of 20 randomly selected antigens. Groups 2, 3, and 4 were immunized with VP30 and VP72, along with 20 randomly selected antigens (different from group 1) for group 2, 10 membrane protein antigens for group 3, and no additional antigens for group 4. All the pigs from four groups were bled before and 10 weeks after immunization.

Sera from infected French pigs. Sera samples of four ASFV infected pigs and two ASFV uninfected pigs were collected from our French collaborators. All these pigs were specific pathogen free (SPF).

Sera from infected British pigs. There are only two sera samples in this group: TQ13 and MI92. Both are from OURT88/3 infected pigs (OURT88/3 is an ASFV isolate). Unlike the French pigs, these pigs were not under pathogen control programs.

Beads TMB ELISA

In order to test our protein made by *in vitro* transcription/translation (IVTT) expression, TMB ELISA were performed as follow procedure: (1). Add ~0.1ug of protein on beads to wells of a MaxiSorp 96-well plate. Remove buffer. Add 100µl of ELISA coating buffer (0.03M NaHCO3, 0.02M Na2CO3). Seal plate, incubate 4ºC overnight. (2). Wash the plate on magnetic stand 3X with 1X PBS + 0.05% Tween-20 or 1X TBS + 0.05% Tween-20. (3). Add 200µl of 3% BSA in 1X PBS to all wells, seal plate, and incubate at 37ºC for one hour. Meanwhile, prepare serum dilutions in 3% BSA in 1X PBS. (4). Wash MaxiSorp plate from step 3 3X on magnetic stand. Then, add 100µl of diluted sera from the deep well plate in step 4 to the MaxiSorp plate to appropriate wells. Each serum dilution should be done in duplicate. Seal the plates and incubate at 37 degrees for 1 hour. (5) Wash MaxiSorp plate 3X on magnetic stand. Add 100µl of 1:3000 dilution of Goat anti-swine IgG (H+L) HRP secondary antibody in 3%BSA in 1X PBS to appropriate wells. Seal plate and

incubate at 37ºC for one hour. (6) Wash MaxiSorp plate 3X on magnetic stand. Add 100µl of TMB (warmed at room temp ~5 min) to all. Allow reaction to proceed until color development is sufficient. (7)100µl of 0.5M HCl to each well to stop color development. (8) Read at 450nm in SpectraMax M5 within 30 min. of stopping reaction.

Construction of LEE structure

Due to the failure of express VP30 protein recombinantly, we switch to another protein expression strategy: *In Vitro* Transcription/Translation (IVTT) system. We also take advantage of the Linear Expression Elements (LEE) technology in our lab to improve the yield of our interested proteins. By LEE technology, we were able to noncovalently link the PCR-amplified open-reading frame (ORF) to a eukaryotic promoter and terminator, which can further used as the template in IVTT reaction(Sykes and Johnston 1999). To construct the LEE for VP 30, we first generated three cassettes: VP30 open reading frame (ORF), promoter and terminator. The 50µl PCR reaction system include: 5.0 µ l 1ng/µl template, 2.5µl 10µM forward oligo, 2.5µl 10µM reverse oligo, 5.0µl 10X Pfu Turbo Buffer, 0.4µl 100mM dNTPs, 1.0µl Pfu Turbo and 33.6µl water. The forward and reverse primers we used to amplify VP30 ORF were ASFV127_F (ggtataggcggaagcgccatggattttattttaaatatatccatgaaaatggag) and ASFV127_R: gtcttcttcgctaatcagtttctgttcaaacattaaatgtaggtgagataaa agcttatttt. The primers we used to amplify the promoter were T7_Trx_F (gcgaaattaatacgactcactatagg) and T7_Trx_R (cctcgacgctaacctggccggta

taggcggaagcgcc). The primers we used to amplify the terminator were T7 Term His ASFV F (gaacagaaactgattagcgaagaagaccatcatcatcattaataaaa gggcg) and T7 Term R ASFV (ctgaaaggAggaactatatccggat). The PCR conditions were: denature 1 minute at 95ºC, then 95ºC 30 seconds/52ºC 30 seconds/72ºC 2.0 minutes for 40 cycles. After confirm the length of the PCR products by running on 0.8%-1.0% agarose (Lonza) gel, we purify remaining product by Qiagen gel extraction kit. Then we did overlapping (recursive) PCR to get the full length LEE cassette for VP30. The 50µl reaction system was: 10.0µl 160fmol ORF (+ water); 1.0µl 160fmol T7 Trx Promoter ASFV (42.35ng); 1.0µl 160fmol T7 Term His ASFV (31.82ng); 5.0µl 10X Pfu Turbo Buffer; 0.4µl 100mM dNTPs; 1.0µl Pfu Turbo and 31.6µl water. The PCR conditions were: denature 1 minute at 95ºC, then 95ºC 30 seconds/52ºC 25 minutes/72ºC 2.5 minutes for 40 cycles. After overlapping the three segments, we amplified the whole cassette by another round of PCR. The amplification PCR reaction systems were: 10.0µl recursive PCR, 4.0µl 10µM F primer: T7 Trx ASFV F new, 4.0µl 10µM R primer: T7 Term His ASFV R, 8.0 µl 5X iProof Buffer, 0.5µl iProof, 0.4µl 100mM dNTPs, 23.1µl water. The PCR conditions were: denature 30 seconds at 98ºC, then 98ºC 10 seconds/58ºC 30 seconds/72ºC 1.5 minutes for 25 cycles. If the PCR product were confirmed by running a test gel, we purify the remaining PCR product by Qiagen PCR purification kit.

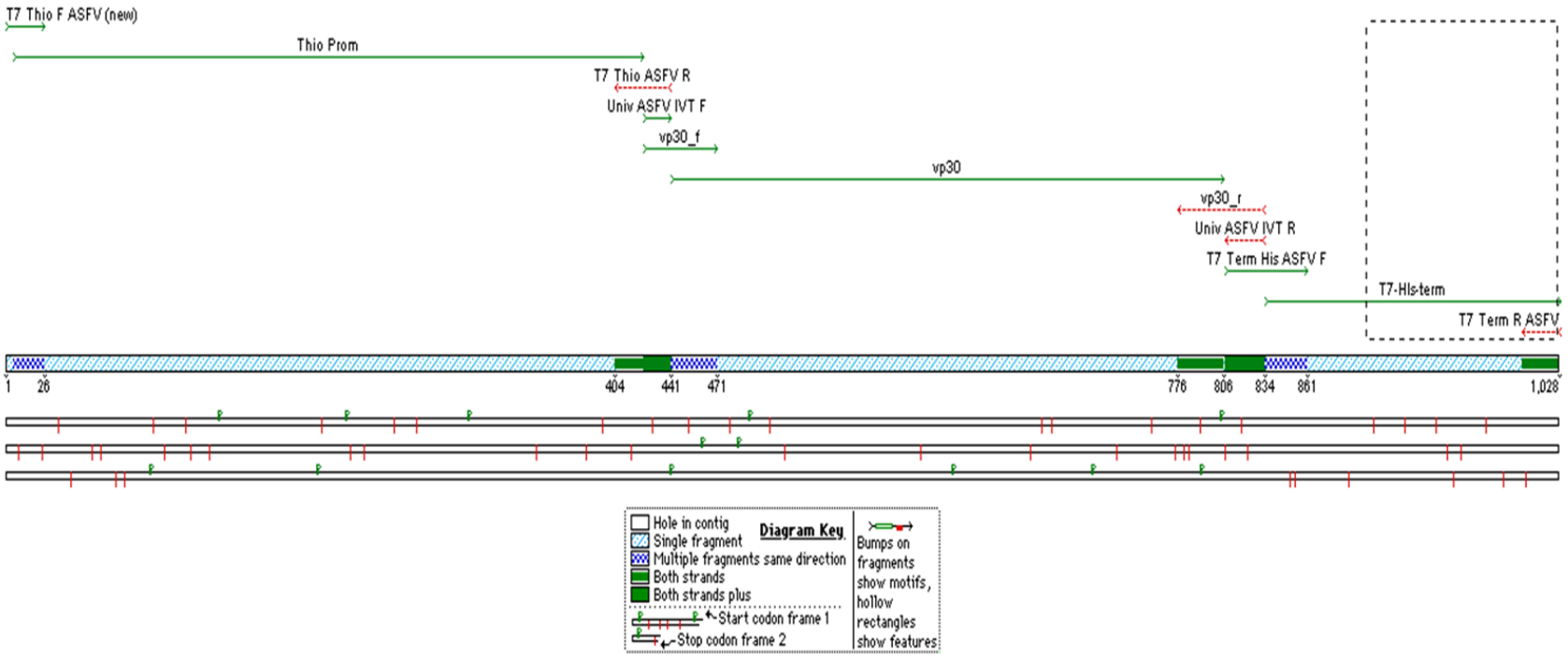


Figure 1. The Strategy of building VP30 LEE construct

Protein Expression in E.coli

The E.coli strain we used was One Shot® BL21 Star™ (DE3) Chemically Competent E. coli from Invitrogen. The basic transformation procedure is: (1) Thaw one vial of competent cells on ice per transformation. (2) Add 5~10 ng of DNA, in a volume of 1~5 µL to the cells and mix gently. (3) Incubate the on ice for 30 minutes. (4) Heat shock the cells by incubating for exactly 30 seconds in the 42°C water bath. (5) Remove it from the 42°C bath and quickly place on ice. (6) Add 250 µL of pre-warmed Super Optimal Broth (SOC) medium. (7) Shake the cells at 37°C for 1 hour at 225 rpm. (8) Plate two different volumes of the transformation reaction onto LB plates containing the appropriate antibiotic. (9) Invert the plates and incubate at 37°C overnight.

Once we got the colonies from the plates, we start to induce the protein by the protocols as follow: (1) Overnight Culture: Pick up one clear colony from a fresh plate and inoculate it into 10 ml LB media with 50µg/ml Carbenicillin. Grow in the Innova 44 Incubator Shaker with 37°C at 250 rpm. (2) Large culture: Use 1 to 100 dilution of the overnight culture to inoculate larger culture of LB media with 50µg/ml Carbenicillin. (3) Allow to grow it at 37°C at 250 rpm. Measure the OD600 every 30 minutes by Eppendorf Bio photometer. Stop growing until the OD600 reach 0.6. (4) Induction: Use the optimized concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the protein for 2 to 4 hours with 37°C at 250 rpm shaking. In our case, the optimized concentration of

IPTG was 1 mM final concentration (5) Collect the cells. Spin the cells at 6000Xg for 10 minutes by using centrifuge tube (Nelgene 3141-0250 Wide mouth, PPCO).

After inducement, we prepared E.coli lysis by follow procedure: (1) Prepare the suspension buffer: 1XPBS, 1.0% Triton X-100, 1 mM PMSF and a protease inhibitor tablet. Then suspend the cell pellet in suspension buffer (5 ml for 200 ml LB culture). (2) Dissolve about 10 mg of Lysozyme in 500ul of PBS and add to the lysis. (3) Incubate at room temp for 15 min with occasional shaking, and then freeze the lysate at -80 ºC for at least 10 min. (4) Thaw the lysate completely at room temp. (5) Repeat Freeze/Thaw two more times and spin the lysate at 27000Xg for 10 min. (6) Collect the supernatant and the pellet separately.

The protein was purifed by using Ni Sepharose™ High Performance from GE Healthcare followed by the manufacturer manual. The chelating group coupled with agarose beads is precharged with Ni^{2+}, which can selectively retain proteins with exposed histidine groups.

Protein Expression in IVTT

To *in vitro* translate VP30 protein, we use Invitrogen kit with following procedure: (1). Wash 25µl M-280 Tosylactivated beads/reaction 3X with 1.0ml 1X PBS. (2). Treat work area and pipettes with RNase/DNase Away cleaner (3). Transfer 25µl of suspended beads to protein low-bind tubes/plate and remove PBS buffer (4). Add 50µl of Invitrogen IVTT master mix (Table 1). (5). Incubate 30ºC in plate in proteomaster/incubator

for 30min. (6). Add 50µl of feed reaction (Table 2). (7). Incubate 30ºC on plate in proteomaster/incubator for 3.5 hour. (8). Wash beads 3X with 0.5ml 1X PBS. Freeze beads for later use here.

Master Mix	(ul)
E. coli SlyD- extract	20.00
2.5X IVPS Rxn Buffer	20.00
50mM AA (-Met)	1.25
75mM Met	1.00
T7 Enzyme Mix	1.00
DNA Temp (500ng)	4.50
50X MiniComplete prot. Inhib.	1.00
RNase Inhib 40u/ul	1.25
Tot Rxn Vol	50.00

Table 1. Invitrogen IVTT Master Mix components

Feed Reaction	
2X IVPS Feed Buffer	25.00
50mM AA (-Met)	1.25
75mM Met	1.00
50X MiniComplete prot. Inhib.	1.00
RNase Inhib 40u/ul	1.25
H2O	20.50
Total Vol	50.00

Table 2. Invitrogen IVTT Feeding Reaction components

M-280 Tosylactivated beads are magnetic beads for small-scale purification. The VP30 coated beads are obtained after mixing M-280 Tosylactivated magnetic beads with the IVTT reaction, and followed by magnetic separation to remove supernatant.

SDS-PAGE

SDS-PAGE gel. NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 10 well (Invitrogen™ # NP0321BOX), NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 12 well (Invitrogen™ # NP0322BOX) and NuPAGE® Novex 4-

12% Bis-Tris Gel 1.0 mm, 15 well (Invitrogen™ # NP0323BOX);The running buffer were prepared by adding 50 ml 20X NuPAGE®MES to 950 ml of deionizer water. The running conditions are: 200 constant voltages for 40 minutes.

Silver stain. We expected the antibodies that eluted from beads will be in very low concentration, thus silver stain might be necessary in order to see the bands. The kit we used for silver stain is Pierce® Silver Stain Kit (Thermo #24612). All solutions were prepared as follow: Fixing Solution: 30% ethanol: 10% acetic acid in ultrapure water; Sensitizer Working Solution: 40µl Sensitizer with 20ml ultrapure water; Stain Working Solution: 400µl Enhancer with 20ml Stain; Developer Working Solution: 400µl Enhancer with 20ml Developer. The procedure summarized as follow: (1) Wash gel with ultrapure water twice for 5 minutes. (2) Fix the gel with Fixing Solution for 15 minutes, twice. (3) Wash the gel with 10% ethanol twice for 5 minutes. Then wash the gel with ultrapure water twice for 10 minutes. (4) Sensitize the gel with Sensitizer Working Solution for 1 minute, and then wash the gel with ultrapure water twice for 1 minute. (5) Stain the gel with Stain Working Solution for 30 minutes. (6) Wash the gel with ultrapure water twice for 20 seconds each. Then develop the gel with Developer Working Solution for 2~3 minutes until bands appear. (7) Stop with 5% acetic acid for 10 minutes.

Depletion of virus specific antibodies from serum

To deplete target antibody, we first prepared the 1/250 dilution sera. For the ELISA experiments, we use 3% BSA in 1X PBS to make the dilution. To prepare depleted sera used to probe 10K random peptide microarray, we use incubation buffer to make the dilution. The incubation buffer was prepared as follow: 5ml of 30% BSA, 25ul of Tween 20, 5ml 10x PBS, 40 ml ddH20. We then added VP30 beads from IVTT reaction that equivalent to certain amount of VP30 protein into the diluted sera in the low protein binding tubes (Eppendorf Protein LoBind Tubes). Then we shake the tube overnight in Rapid Translation System Proteomaster at 37°C at 900 rpm, or in eppendorf Thermomixer R at 37°C at 1200 rpm. We spin down the beads, collect the antibody-depleted sera and keep the beads at 4°C the next day.

To elute the bound antibodies from the VP30 on the beads, we used the procedure as follow: (1) prepare elution buffer by adding 11.1g Glycine-Hcl to 800ml dH2O, then adjusting pH from 3.8 to 5 and bring volume to 1liter with dH2O. (2) Elute bound antibodies with glycine buffer, pH=3.8 three times by mixing/vortexing for 5-10 minutes, then elute bound antibodies with glycine buffer, pH=5 three more times by mixing/vortexing for 5-10 minutes. (3) Collect the supernatant into a new tube and add 1/10 the volume of 2M Tris buffer, pH=8.0 to neutralize the antibody in order to prevent denaturation of antibodies.

Probing CIM 10K ver2 random peptide microarray with ASFV sera

Tecan automated processing. We first prepared the reagents as follow: (1) Blocking Buffer: 5 ml of 30% BSA, 6.9 ul of Mercaptohexanol, 25 ul of Tween 20, 5 ml 10x PBS, 40 ml ddH20; (2) Incubation Buffer: 5 ml of 30% BSA, 25 ul of Tween 20, 5 ml 10x PBS, 40 ml ddH20. (3) Sera: Sera were prepared by 1/500 dilution of incubation buffer that prepared in (2). (4) Prewash buffer: 7.33% acetonitrile, 33% isopropanol and 0.55% triflouroacetic acid in nanopure water. (5) Secondary antibody: Goat Anti-Swine IgG (H+L)-BlOT from BioFX (Cat No. 120-120-06) was diluted to 5 nmol by using incubation buffer prepared in (2). (6) Tertiary antibody: Streptavidin Alexaflour 647 from Invitrogen was diluted to 5nmol by using incubation buffer prepared in (2).

We prepared the slides by following procedures: (1) Place slides in a slide washer occupying only every other space to ensure good solvent flow. (2) Submerge slides in wash buffer and place on the rotating platform in the chemical fume hood that is set for slow speed (approximately setting 4) for 5 min. (3) Manually wash 1x in Tris Buffer Saline Tween20 (TBST) buffer. (4) Manually wash 1x in ddH2O. (5) Spin and dry the slides at 1500 rpm for 5 min.

We then use Tecan machine (HS 4800™ Pro) to automated process the slides. The procedures we used on Tecan were basically included: (1) Blocking the 10K microarray surface by incubating with blocking buffer for 30 minutes. (2) Probe 10K random peptide microarray with pig sera. (3) Probe 10K random peptide microarray with anti-swine secondary antibody

for one hour. (4) Fluorescently labeling the secondary antibody by the streptavidin conjugated Alexaflour dye for one hour.

Optimize the secondary antibody concentration. Since this is the first time in our lab to use anti-swain secondary antibody on Tecan. Optimize its concentration is necessary in order to get good immunosignatures of 10K microarray. Three different secondary antibody concentrations: 2 nmol, 5 nmol, 10 nmol, have been tested and their dynamic ranges were measured on Genepix. The concentration of the best dynamic range and the cleanest background was selected as the standard concentration for all the following experiments.

Statistical analysis

Principle component analysis (PCA). It is easy to plot a set of data with only two variables and visualize the correlation between them. However, in our experiment, the signals of thousands of peptides were measured across many different variables such as treatments or time points. With so much data organized in a multi-dimensional matrix, it becomes very challenging to make a visual inspection of the relationship between peptides and variables. One way to simplify the data is to reduce its dimensionality (Fig. 2). Several data decomposition techniques are available for this purpose, among them Principal Components Analysis (PCA). By performing a covariance analysis between factors, PCA can reduce the data to a few dimensions. PCA can also transform the data to a new set of variables (the principal components) and summarize the

features of the data. Since PCA contains most of the variants in the data, it can also be used in cluster analysis (Yeung and Ruzzo 2001).

In this study, PCAs were performed by Genespring GX 7.3 from Agilent Technologies to find relevant components, or patterns, across samples or conditions. All PCAs were displayed in a two dimensions scatter plot view, with the two most important components on each axis. Each dot on the PCA plots represents a sera sample. The distance between two dots on the PCA plot can be interpreted as the differences of the immune responses.

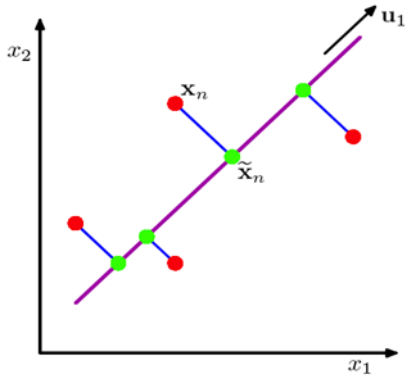


Figure 2. Principle component analysis seeks a space of lower dimensionality. The projection of the data points (red dots) onto the lowest dimensionality (magenta line) maximizes the variance of the projected points (green dots).

One way ANOVA test and Student t-test. In our study, one way analysis of variance (ANOVA) was used to determine if one given factor, such as with immunization or without immunization, had a significant effect

on the immune reactivties against peptides across any group that was studied. This model could be written as:

$$Y_{ij} = \mu_i + \varepsilon_{ij};\ i = 1, ..., r,\ j = 1, ..., n_i.$$
$$\varepsilon_{ij} \overset{i.i.d.}{\sim} N(0, \sigma^2)$$

The assumptions for this model are: (1) Corresponding to each factor level, there is a probability distribution of responses. (2) The probability distribution of all groups is normal. (3) Each probability distribution has the same variance. (4) Responses are statistically independent. The null hypothesis is that there is no difference in the mean peptide expression intensities across the groups we tested.

A significant p-value from the 1-way ANOVA test would indicate that a peptide was expressed differently by at least one of the groups analyzed. In our study, the p-value cut-off was set to 1e-4. However, if there were more than 50 peptides that passed the restriction, only the top 50 peptides with the lowest p values were picked. We did not see much difference when using more than 50 peptides to cluster a heat map or to plot a PCA map. 50 peptides can hold enough representiveness but also minimize the noise. Some of the peptides may be falsely considered as statistically significant. To reduce overall error rate and the false discovery rate, we performed multiple testing corrections (MTC) after ANOVA or t test. The Benjamini and Hochberg MTC methods were used. Though they are not the most stringent MTC, they offer a good balance between discovering

statistically significant differences in peptide expression and protecting against false positives (Kim, Dougherty et al. 2002).

GLAM2 motif and Bepipred linear epitope prediction. Gapped Local Alignment of Motifs (GLAM2) is a tool for discovering motifs in a group of DNA or protein sequences. We use GLAM2 to find the common motifs across a set of peptides we selected. The advantage of using GLAM2 is its ability to discover gapped motifs. GLAM2 will not only find the best motif in one run, but also performs it ten times to confirm its results.

Further, we used the Bepipred linear epitope prediction tool (http://tools.immuneepitope.org/tools/bcell/iedb_input) to predict antibody epitopes by the protein sequence. BepiPred can predict the location of linear B cell epitopes by using a combination of hidden Markov models and a propensity scale method. The propensity scales for each of the 20 amino acids includes hydrophilicity, flexibility, accessibility, turns, exposed surfaces, polarity and antigenic propensity of polypeptides chains.

Once we got the predicted epitopes for the original protein, we compared them with the common motifs that were found among the selected peptides to see if they were alike.

Chapter 5

RESULTS

The antibody reactivity profiles of immunized and unimmunized pigs against random peptide microarray can be distinguished

Because of the complexity of an immune-profile comprised of antibody responses to such a large number of peptides on our array, it was difficult to compare the profiles without data reduction. To reduce dimensions, 50 out of the 9,786 peptides were selected as displaying the most significant differences in reactivity levels between immunization groups using a t test (p value $\leq$ 1.39e-11). We clustered 40 sera samples based on these 50 peptides by Genespring 7.3. The hierarchical clustering algorithm Genespring 7.3 uses is the 'centroid' clustering method. In this method, the distance between two clusters is the distance between the averages of the data points under one branch and the averages of the data points under another. As shown on the heat map (Fig. 3(A)), only two pre-immunization pigs were misclassified to the post-immunization group. The accuracy of grouping was 95%. From the PCA plot (Fig. 3(B)), although the two clusters were not separated very well by distance, we can still pick out the clusters of pre-immunization group and post immunization group. This reveals the ability of these 50 peptides to distinguish the different immunosignatures between ASFV antigen-immunized and unimmunized pigs.

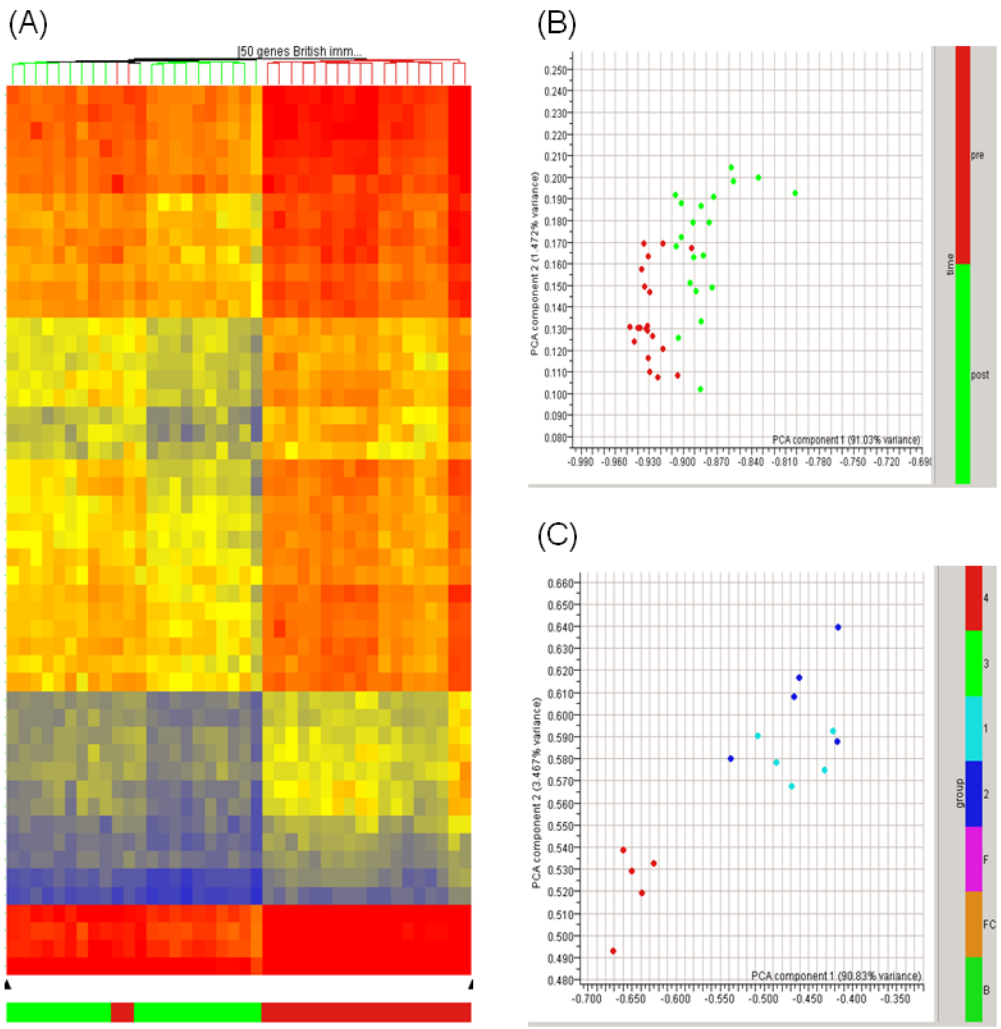


Figure 3. Immunosignatures of British pigs from immunization groups. (A) Heat map generated by 50 peptides that were selected to distinguish between immunized pigs and umimmunized pigs. Blue on the heat map indicates the lowest expression level (the weakest binding between antibodies and peptides), and red indicates the highest expression level. The color bar at the bottom indicates the classification of pigs (Green represents immunized pigs and red represents unimmunized pigs). (B) Principle Component Analysis (PCA) plot showing all the immunized and

unimmunized pigs in (A). (C) PCA plot showing the clusters of immunized pigs from different groups.

Earlier, we hypothesized that the pigs immunized with different antigens would have different immunosignatures. To test our hypothesis, 19 peptides were selected by one way ANOVA test that most significantly distinguish 4 different post-immunization groups (p value≤1e-4). One way ANOVA test was used instead of t test because more than two groups of samples were involved in our comparison. We plotted a PCA to cluster those four groups. Interestingly, we found that group 4 pigs, which were immunized with two known immunodominant antigens, were separated from group1 and group 2, both of which were immunized with 20 unique antigens in addition to the two used for group 4 (to make a cleaner look of the PCA plot, group 3 was ignored due to its random distribution). To rationalize this separation between group 4 and groups 1&2, we suggest that the immunosignatures of the two immunodominant antigens were not the only antibody reactivites elicited by immunization with the pools of other antigens.

The antibody reactivity profiles of infected and uninfected French sera against random peptide microarray can be distinguished

To determine if we can differentiate the immuno-profile of infected French pigs from the immuno-profile of the uninfected French pigs, seven peptides were selected using t test (p value≤1e-4). Using the heat map display, we can compare the reactivity profiles of these peptides, which also classified the two groups of pigs perfectly (Fig. 4(A)). The PCA plotted by these peptides also confirmed that four infected pigs were well separated from uninfected pigs (Fig. 4(B)). These seven peptides do not overlap with the 50 peptides we selected for distinguishing antigen-immunized sera from unimmunized sera. We reasoned that a viral infection may elicit different immuno-responses from the immunizations with a limited number of antigens and such differences influenced the reactivity profiles against peptides on the array. There could be some other important antigens that play important roles during the viral infection. The strong expression level of these seven peptides may reveal the reactivities of those undiscovered antigen responses.

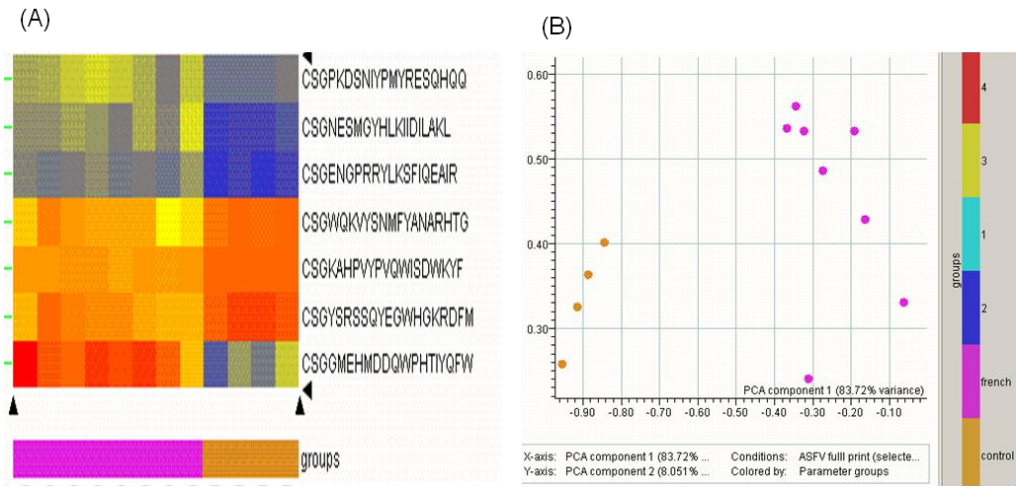


Figure 4. Immunosignatures of vial infected French pigs. (A) Heat map generated by seven peptides that were selected to distinguish between infected French pigs and uninfected French pigs. The color bar at the bottom indicates the classification of pigs (brown represents vial infected French pigs and purple represents uninfected French pigs). (B) PCA plot showing the same pigs in (A).

The antibody reactivity profiles of infected and uninfected British pigs against random peptide microarray can be distinguished

The sera from British infected pigs were received after the first round of experiments which tested the sera from antigen immunized pigs and sera from vial infected French pigs. These British infected pigs were not raised in a controlled, pathogen-free environment. Therefore, unlike specific pathogen free (SPF) pigs from France, British pigs likely to have been exposed to other pathogens, so would be expected to have more complex immunological reactivities and thus more similar to the wild pigs and the pigs in food industry. Therefore, it is meaningful to test the French sera samples separately from the British sera samples. Since we don't have pre-infection sera samples, the unimmunized British sera were assayed as the controls. 50 peptides were selected as displaying the most significant differences in reactivity levels between these two groups using a t test (p value≤6.67e-20). As shown on the heat map that generated by these 50 peptides, we can clearly see that the reactivity profiles of these peptides were distinct between the infected pigs and the control pigs (Fig. 5 (A)). Also, the classifications of the two groups were perfect. From the PCA plot, a significant separation between these two groups can be observed. Although it is insufficient to have tested only two samples before making a conclusion, the immnosignatures captured with the 10K random microarray are consistent and reproducible for pigs with different immune histories.

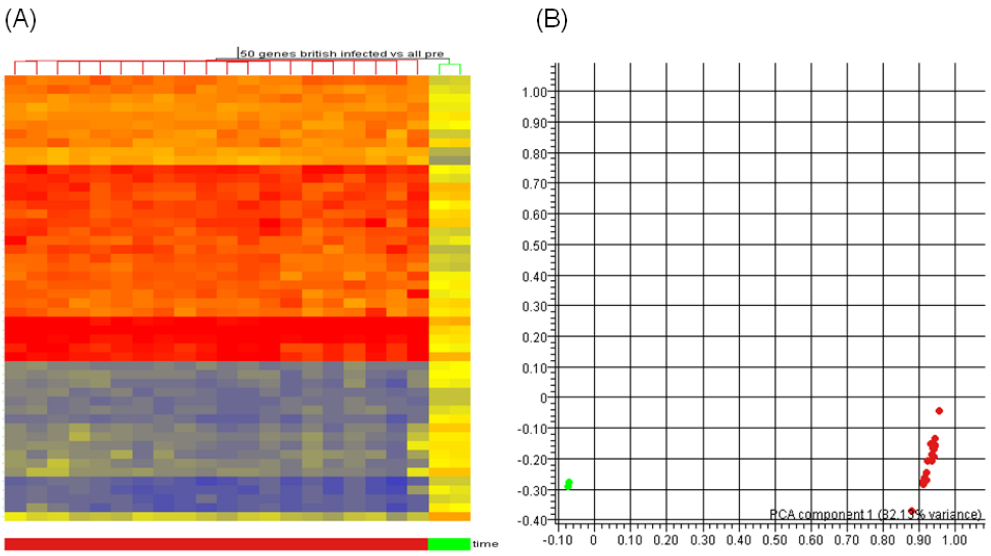


Figure 5. Immunosignatures of vial infected British pigs. (A) Heat map generated by 50 peptides that were selected to distinguish between infected British pigs and unexposed pigs. The color bar in the bottom indicates the classification of pigs (Green represents vial infected British pigs and red represents uninfected pigs). (B) PCA plot showing the same pigs in (A).

The antibody reactivity profiles of all infected and all uninfected sera against random peptide microarray can be distinguished fairly well

We combined the expression profiles from infected British sera and infected French sera and endeavored to determine whether we can segregate them from unexposed sera. We picked the 50 peptides that most significantly distinguished these two groups by a t test and clustered these peptides to make a heat map (p value ≤ 2.1e-15). Using the heat map display, we can see two distinctive profiles. Although a few of the unexposed pigs were misclassified, most of the pigs were correctly clustered to its own group. In general, the profiles from unexposed pigs had lower expression levels compared to the profiles of infected pigs. This may indicates that the antibodies from the infected pig sera display higher reactivities to these peptides. The PCA plot also confirmed the separation between infected and uninfected pigs (Fig. 6(B)). We also compared these 50 peptides with the 50 peptides selected to distinguish British infected sera and unexposed sera. We found only one common peptide. The possible explanation could be: although British infected pigs and French infected pigs show similarities in their binding patterns, their highest binding reactivities are distinct.

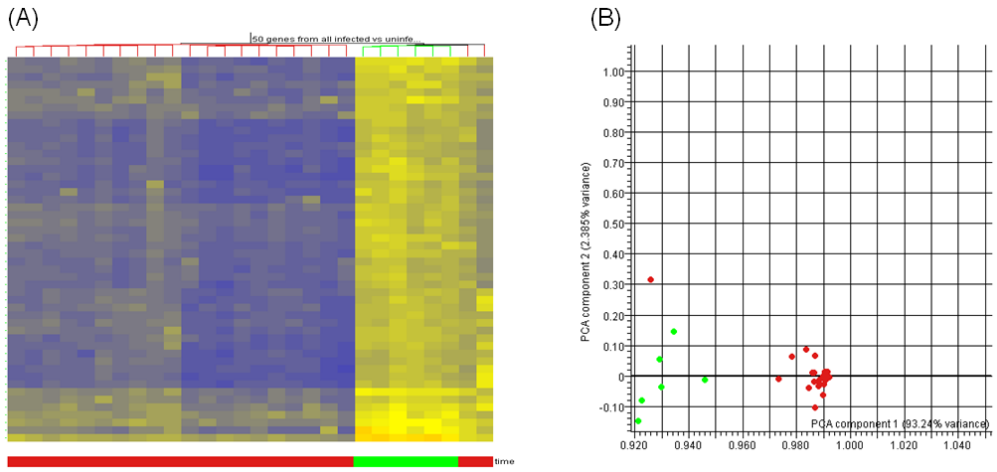


Figure 6. Immunosignatures of vial infected pigs. (A) Heat map generated by 50 peptides that were selected to distinguish between all infected pigs and all unexposed pigs. The color bar at the bottom indicates the classification of these pigs (Green represents vial infected pigs and red represents uninfected pigs). (B) PCA plot showing the same pigs in (A).

The antibody reactivity profiles of ASFV or ASFV antigen exposed and unexposed sera against random peptide microarray can be distinguished

In this experiment, immunosignatures were gathered from all the pigs. Although these pigs had different origins and were exposed to different immunogens, the most obvious difference that can separate them is that some pigs were exposed to ASFV – either ASFV antigens or the attenuated virus – while the others were not. We selected 50 peptides that have the best p values in the t test and clustered them into a heat map (p value ≤ 3.86e-10). Using the heat map display, we can identify two major patterns of antibody reactivity profiles. Although the classification of pigs was not perfect, 85% of the immunosignatures were correctly grouped to its own class. We also noticed that the pattern of reactivity profiles here are very similar to the immunized British pigs. The PCA in Fig. 7(B) and Fig. 7(C) were plotted by the same 50 peptides we selected. Although the separations were not very clear, all the pigs tended to aggregate to their own group, especially the unexposed pigs. The highly concentrated distribution of unexposed pigs on the PCA reveals the fewer varieties in immunosignatures, which is immunogically reasonable. On the same PCA, we colored the pigs by their immunization strategies in order to see if we could identify the subgroups of exposed pigs. Although the 50 peptides were not meant to identify these subgroups, we still found the British infected pigs have obvious separation from other exposed pigs.

French infected pigs also separate from other subgroups to a certain extent. However, the four groups of British antigen-immunized pigs could not be separated. This may indicate that the immunosignatures of viral infected pigs are more diverse than the immunosignatures of antigen immunized pigs. Also, undivided subgroups of immunized pigs may suggest that there are some common reactivites that are dominant over the distinct reactivities. Such reactivites might be recognizing VP30 and VP72, or the recombinant vaccinia virus vectors that were used to boost the immunizations.

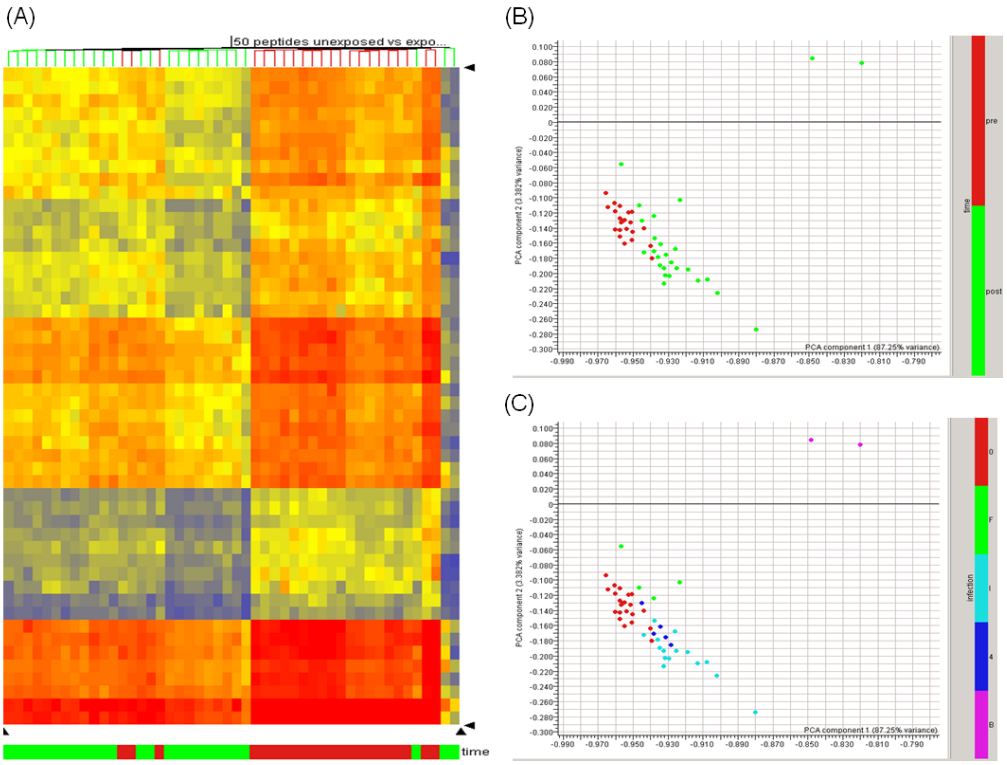


Figure 7. Immunosignatures of ASFV/ASFV antigen-exposed pigs. (A) Heat map generated by 50 peptides that were selected to distinguish between ASFV/ASFV antigen-exposed pigs and unexposed pigs. The

color bar at the bottom indicates the classification of the pigs (Green represents the ASFV/ASFV antigen-exposed pigs and red represents the unexposed pigs). (B) PCA plot showing the same pigs in (A). The red dots represent unexposed pigs and the green dots represent the ASFV/ASFV antigen-exposed pigs. (C) PCA plot showing the same pigs in (A). Red dots represent ASFV/ASFV antigen-exposed pigs, green dots the vial infected French pigs, purple dots the vial infected British pigs, blue dots the group 4 pigs, and the cyan dots all the pigs from group1, group2 and group 3.

The antibody reactivity profiles of VP30/VP72-immunized pigs and ASFV or ASFV antigen-unexposed pigs are poorly distinguished

British pigs from group 4 have been exposed to VP30 and VP72. To test the sensitivity of our 10K random peptide microarray, we compared group 4 immunosignatures with all unexposed immunosignatures. Unlike most of the other experiments that hundreds of peptides can pass the t test, only 14 peptides passed the t test (p value ≤ 1e-4), which reveals that there are a smaller number of differences between immunosignatures from group 4 pigs and immunosignatures from unexposed pigs. Using the heat map display, we could not see clear patterns of the expression profiles, although the classifications of the pigs were fairly accurate (90%). On the PCA plot, the unexposed pigs were scattered and there was no clear separation between immunized group and unexposed group. This result may confirm the assumption we suggested earlier: VP30 and VP72 may not be the antigens that elicit the dominating antibody responses in the context of antigen as opposed to infection. Other unrevealed ASFV antigens, or the vaccinia virus that used to boost the immune-response, may elicit stronger B cell response than VP30 and VP72.

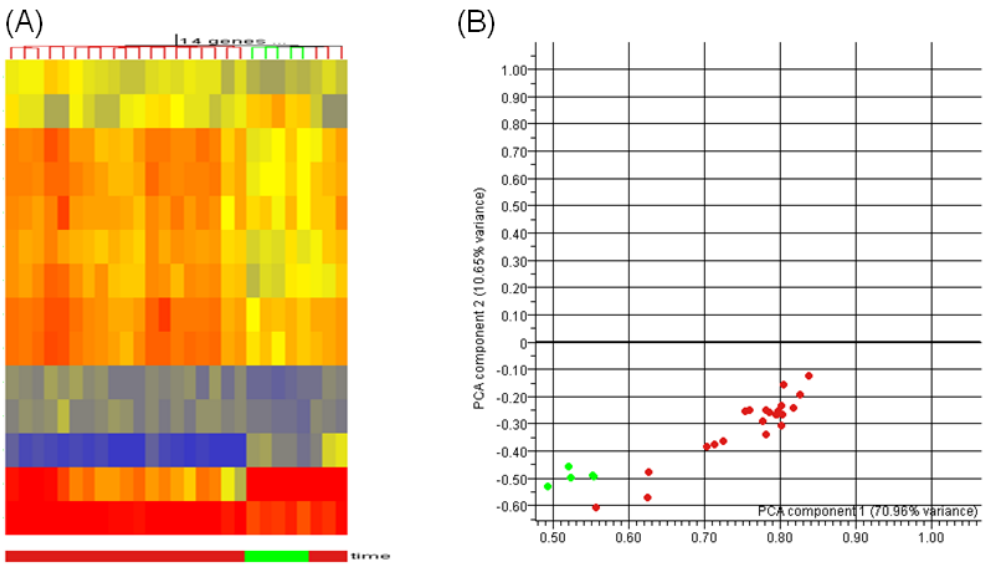


Figure 8. Immunosignatures of VP30/VP72 immunized pigs. (A) Heat map generated by 14 peptides that were selected to distinguish between VP30/VP72 immunized pigs and all unexposed pigs. The colored bar at the bottom indicates the classification of the pigs (Green represents VP30/VP72 immunized pigs and red represents unexposed pigs). (B) PCA plot showing the same pigs in (A).

The antibody reactivity profiles of vaccinia-infected and ASFV-infected British sera against random peptide microarrays are well separated

Group 4 pigs were immunized with only two antigens, and followed by vaccinia boost. From the analysis of the earlier experiments, we have reason to believe that VP30 and VP72 may not be the cause of the dominant immunoresponses in group 4, which leaves vaccinia as an obvious suspect. If such hypothesis is true, we may assume group 4 as a vaccinia-infected group. Under such circumstance, we could compare the immunosignatures of group 4 to the immunosignatures of ASFV infected British pigs in order to see if our 10K random peptide microarray could differentiate two similar, but different, viral infections. To make this experiment more relevant, we removed the peptides that may represent the anti-VP30 antibody response from the selection pool (all 9, 786 peptides). We selected 44 peptides that could distinguish between these two groups from the remaining peptides using the t test (p value ≤ 1e-4). From the heat map generated by these 44 peptides, we can clearly see two distinct patterns of reactivity profiles. Also, the classifications of the tested pigs were perfect. The PCA map also shows clear separation between the two groups without any misclustering. The vaccinia and ASFV are both large double strained DNA viruses, and share a similar virus structure and replication strategies. If our 10K random peptide microarray can differentiate between the immunosignatures of these two

similar viruses, it is also feasible that our microarray could differentiate ASFV from other viral infections. Thus, the 10K random peptide microarray has the potential to be a diagnostic for ASF.

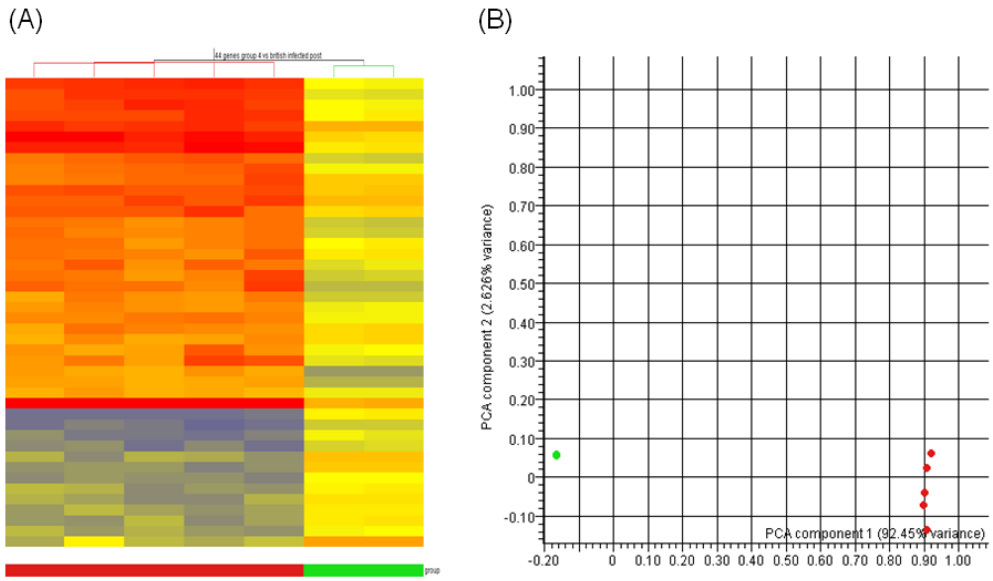


Figure 9. Immunosignatures of vaccinia-infected pigs and ASFV infected British pigs. (A) Heat map generated by 44 peptides that were selected to distinguish between vaccinia-infected pigs and ASFV infected British pigs. The color bar at the bottom indicates the classification of pigs (Green represents ASFV infected British pigs and red represents vaccinia-infected pigs). (B) PCA plot showing the same pigs in (A).

Protein expression and purifications

VP30 expression in IVTT reaction. The VP30 LEE was successfully constructed by assembling three cassettes: Trx_Prom (promoter cassette, 422 bp), VP30 ORF (615 bp), and T7_His_Term (terminator cassette, 222 bp). The full length LEE is 1259 bp. We confirmed the full length LEE on an agarose gel (Fig. 10).

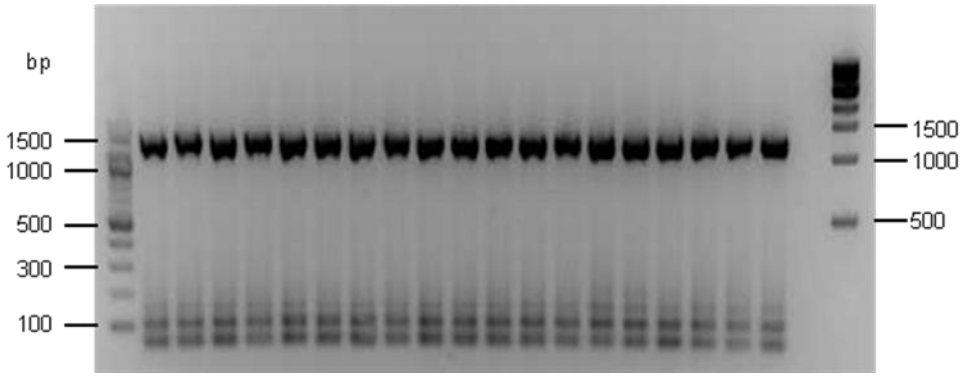


Figure 10. The full length construct of VP30 LEE on 1.0% agarose gel

Once the VP30 LEE was constructed, we started to translate VP30 LEE in an IVTT reaction. We set up twenty 100µl IVTT reactions in order to get enough protein for the ELISA and depletion experiments. After mixing M-280 Tosylactivated magnetic beads with the IVTT reaction, followed by magnetic separation to remove supernatant, VP30 coated magnetic beads are obtained.

All of the VP30 coated magnetic beads were combined into one tube and resuspended in PBS buffer. VP30 concentration was measured by running a SDS-PAGE gel. We prepared a series of protein standards by loading different amounts of BSA (1µg, 2µg, 5µg and 10µg) on the same

gel. By comparing with the BSA standards, the concentration of the final product was roughly 0.5~0.8 μg/μl (Fig. 11). We then stored the VP30 protein at 4ºC.

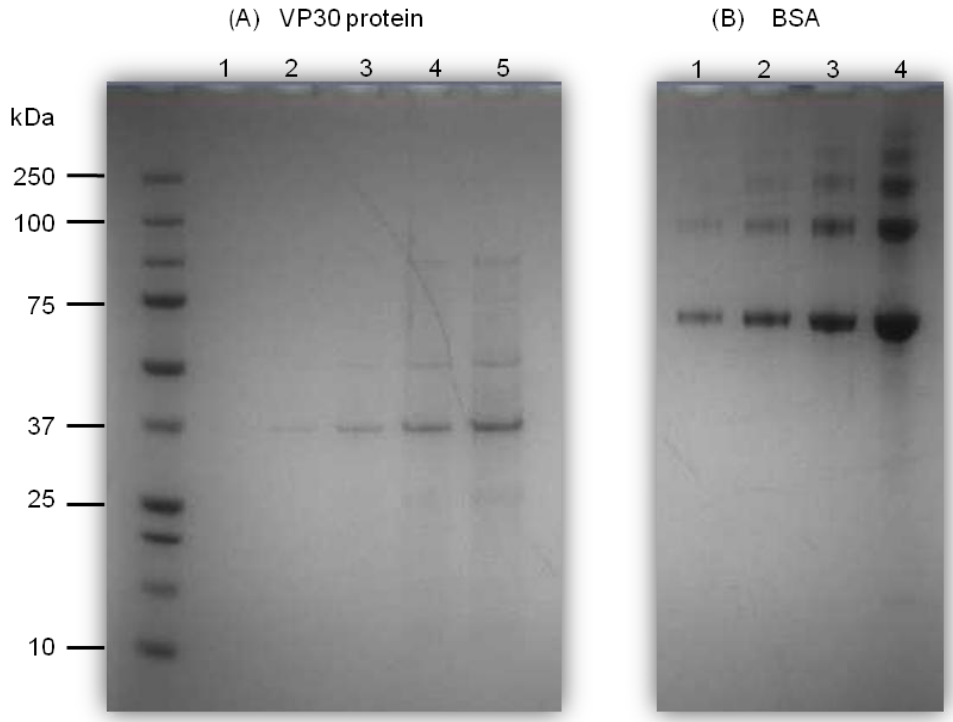


Figure 11. SDS-PAGE gel of VP30 protein. (A) VP30 protein by IVTT expression: Lane 1: 0.5μl of the 50μl final product. Lane 2: 1μl of the 50μl final product. Lane 3: 2μl of the 50μl final product. 4: 5μl of the 50μl final product. 5: 10μl of the 50μl final product. (B) Assessed yield of VP30 protein by loading different amount of BSA. Lane 1: 1μg BSA. Lane 2: 2μg BSA. Lane 3: 5μg BSA. Lane 4: 10μg BSA.

Protein expressions in E.coli. We decided to use thioredoxin (trx) as the control protein since it could be made recombinantly and thus be cost efficiently. We used the empty pET 32 b+ vector as the template. The

thioredoxin protein itself is about 13 kDa, but with the additional tags that are part of the pET 32b+ plasmid such as the S and His tags, the total weight of the entire control protein is ~20kDa. Following the procedure we described earlier, we successfully induced the control protein in the BL21 E.coli by IPTG (Fig. 12).

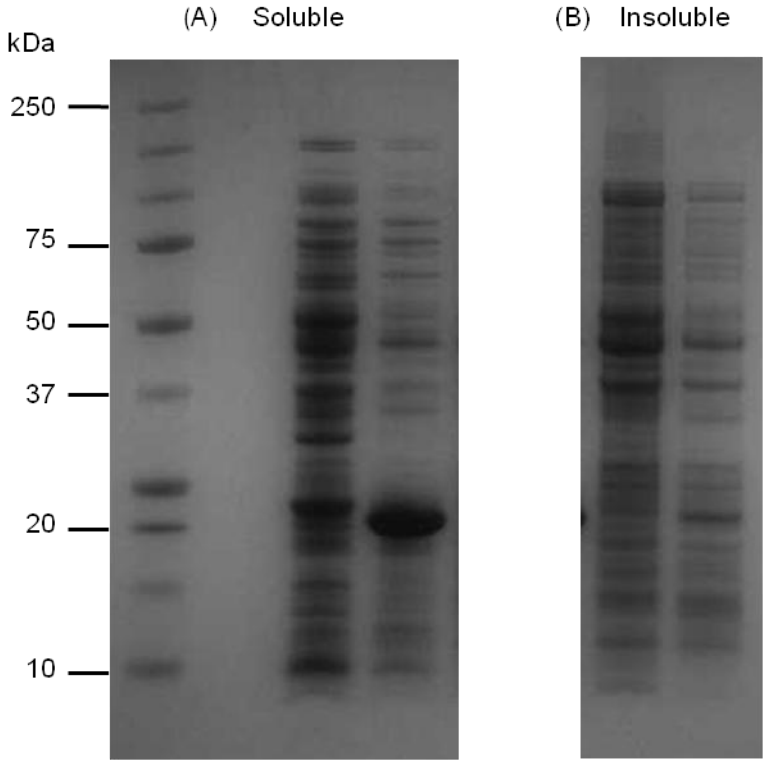


Figure 12. The control protein was expressed in E.coli. (A) Soluble E.coli secretion. Lane 1: Soluble proteins before inducement. Lane 2: Soluble proteins after inducement. (B) Insoluble E.coli secretion. Lane 1: Insoluble proteins before inducement. Lane 2: Insoluble proteins after inducement.

Purification of the control protein. To purify the control protein from E.coli extract, we performed histidine-tagged protein purification. The Ni Sepharose™ High Performance beads from GE healthcare were used to

selectively retain the histidine-tagged thioredoxin. Following the procedure provided by the manufacturer, we successfully purified the control protein. By comparing with BSA standards, we also evaluated the concentration of the purified protein. The concentration of the protein from the first, second, and third washes were 1.2mg/µl, 0.6 mg/µl, 0.24 mg/µl, respectively.

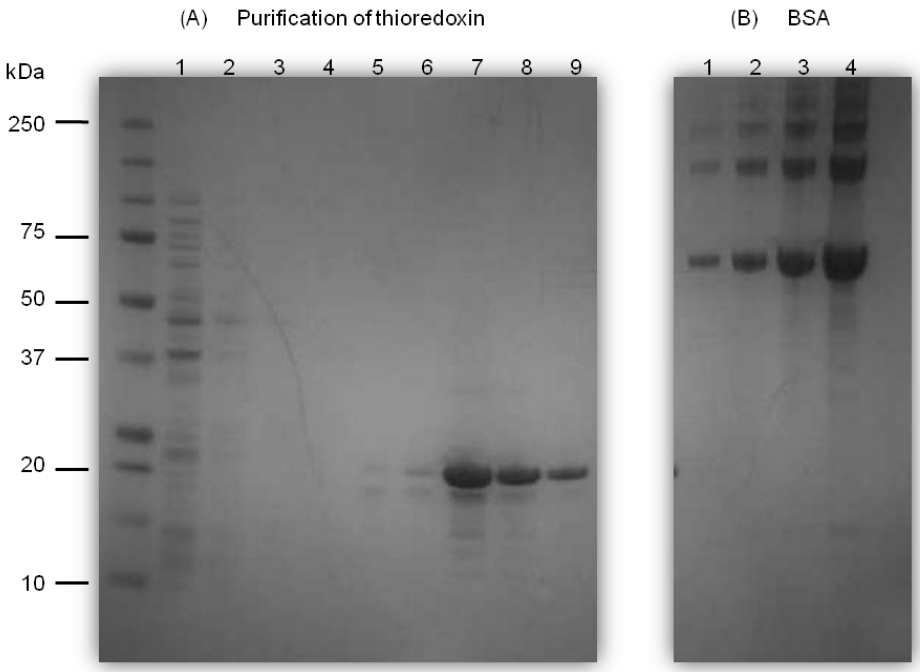


Figure 13. SDS-PAGE gel of the purified Thioredoxin protein (A) The flow through from the purification and the purified Thioredoxin protein. Lane1: Flow through from binding buffer. Lane 2: Flow through from the first wash. Lane 3: Flow through from the second wash. Lane 4: Flow through from the third wash. Lane 5: Flow through from the fourth wash. Lane 6: Flow through from the fifth wash. Lane 7: Thioredoxin from the first elution. Lane 8: Thioredoxin from the second elution. Lane 9: Thioredoxin from the

third elution. (B) Assessed thioredoxin yield by loading different amounts of BSA. Lane 1: 1μg BSA. Lane 2: 2μg BSA. Lane 3: 5μg BSA. Lane 4: 10μg BSA.

ELISA test for using VP30 protein as the coating reagent

To validate the VP30 protein we made by IVTT, TMB ELISA was performed. Both the VP30 and control proteins were coded onto the 96 well plates. Both pre-immunization serum and post-immunization serum of two individual pigs (395 and 403) were selected as the test sera. The ELISA result is shown in the Fig. 14. The anti-VP30 antibody titer from both pigs increased about four-fold after immunization. This is consistent with the former ELISA test. The titer of the control groups, however, did not show many differences between pre-immunization sera and post-immunization sera, which indicates the antibody titer we detected in the test group was specific.

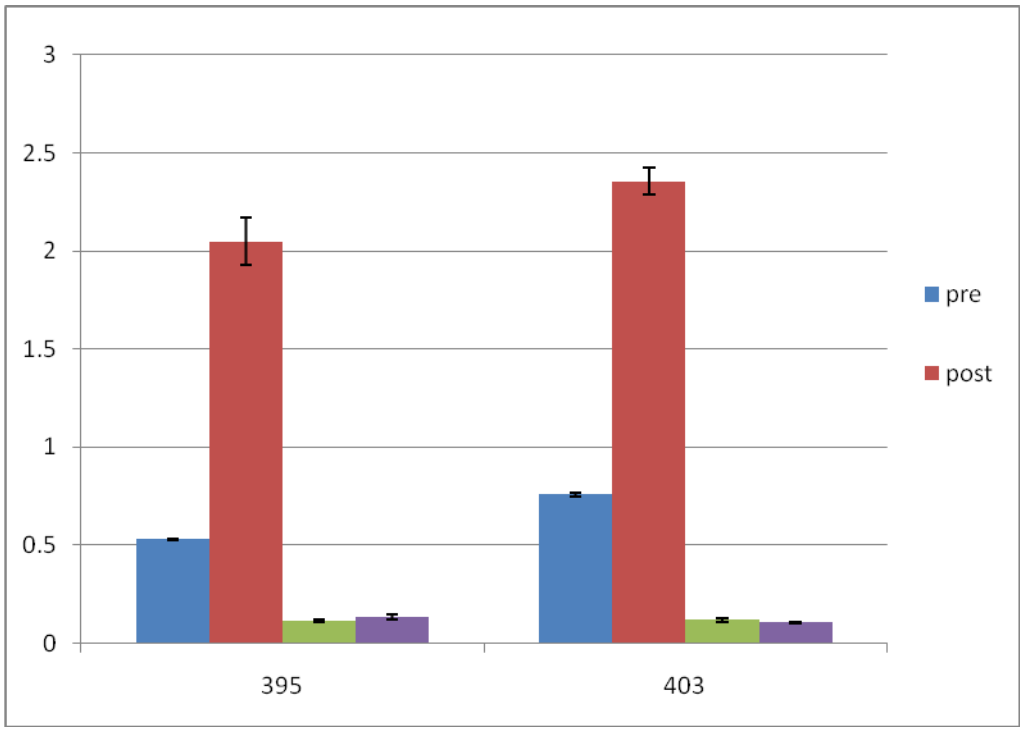


Figure 14. ELISA result for VP30 validation. Blue & Green bar: Pre-immunization serum. Red & purple bars: Post-immunization serum. Both green and red bars represent the titer from VP30 coded wells. Both green and purple bars represent the titer from thioredoxin coded wells (controls).

ELISA test for anti-VP30 depleted serum

Once the identity of VP30 protein made by IVTT reaction was confirmed, we began performing anti-VP30 antibody depletion tests. First, we wanted to test whether the VP30 protein we made by IVTT could pull down the anti-VP30 antibodies. Sera from pig 395 were chosen in the experiment for its high anti-VP30 antibody titer. Three different amounts of VP30 proteins were added to 1/250 diluted post-immunization serum of pig 395. After overnight incubation with constant shaking at 37 ºC, we performed

an ELISA test for these antibody-drained sera. From the ELISA data (Fig. 15), the depletion process drastically decreased the titer of anti-VP30 antibodies to a level even lower than that of the pre-immunization serum. Thus, we believe the depletion was thorough and complete. However, adding more than 5 μg of protein did not make an obvious difference in the matter of titer changes. Three samples of British infected sera were also tested in this ELISA. All three sera showed high anti-VP30 antibody activity.

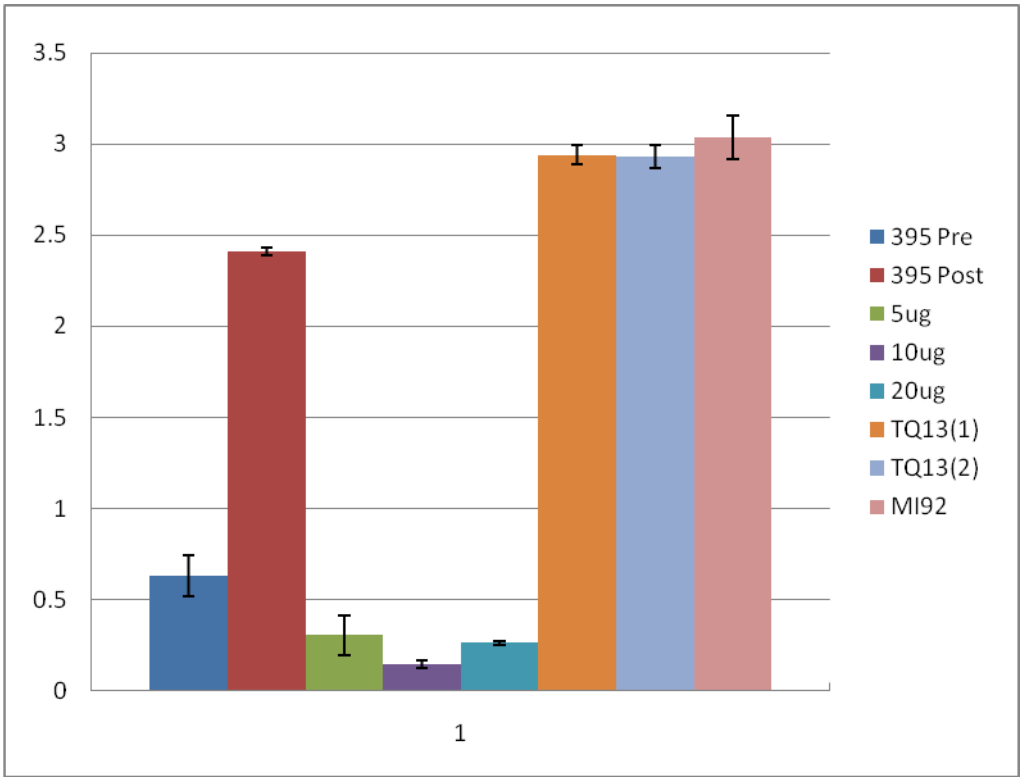


Figure 15. ELISA results from the first depletion test. “395 pre” is nondepleted pre-immunization serum of the pig 395. “395 post” is the nondepleted post-immunization serum of the pig 395. “5μg”, “10μg” and “20μg” represent the sera depleted by the corresponding amount of VP30 protein. “TQ13” and “MI92” are the samples of British infected pig sera.

ELISA test to determine the VP30 quantity required for anti-VP30 antibody depletion

Although we already confirmed that the VP30 protein we made can successfully deplete anti-VP30 antibodies, the optimal VP30 dosage we should use remains uncertain. The overdose of VP30 may cause unspecific binding and pull down antibodies that are unrelated to VP30 during the depletion. To avoid such a situation, we tried to determine the minimum dosage of VP30. We depleted post-immunization pig 395 sera with five different amounts of VP30 protein: 0.1μg, 0.2μg, 0.5μg, 1 μg and 2 μg. We then tested the anti-VP30 antibody titer of these sera, and plotted the data as seen in Fig.16. The very first thing we noticed from the data was the significantly larger standard deviations among replicates. We believe these deviations are the result of unevenly coated wells. In the coating process, we noticed the beads holding VP30 were clogged and could not be suspended evenly. After remaking the VP30 proteins and using the freshly prepared VP30 in subsequent depletion tests, we found the standard deviations were back to normal. From this test, we also found that the anti-VP30 antibody titer tends to increase as we decrease the dosage of VP30. The changes, however, were not very significant.

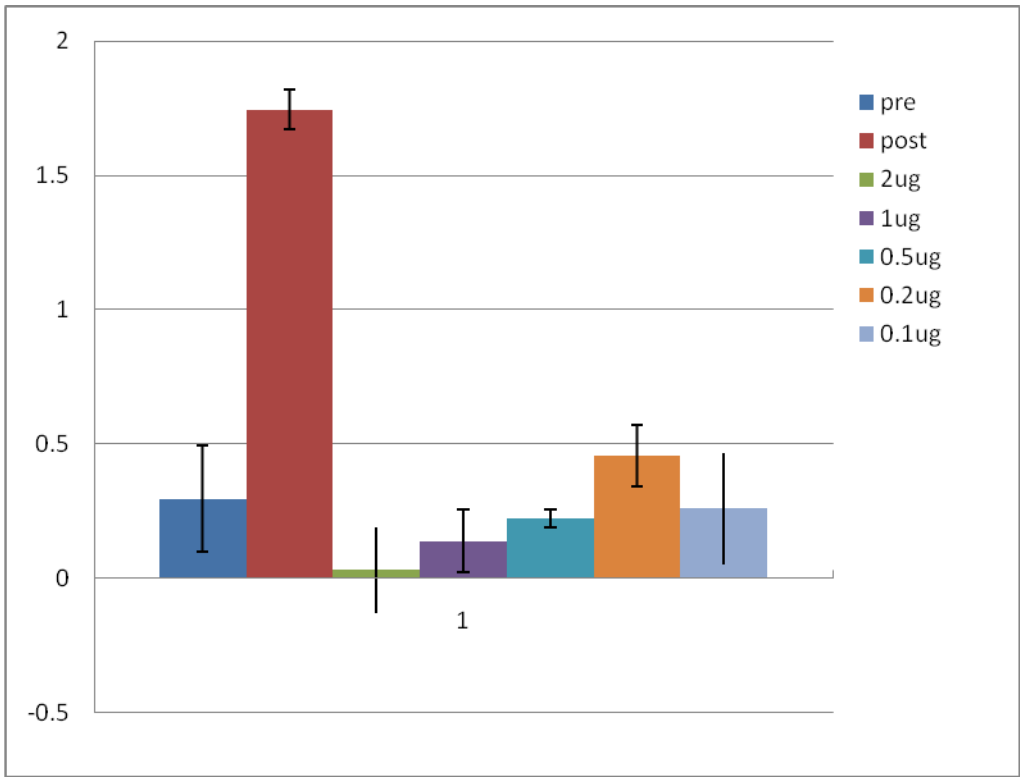


Figure 16. ELISA results from the second depletion test. The “pre” is nondepleted pre-immunization serum of the pig 395. The “post” is the nondepleted post-immunization serum of the pig 395. “0.1 µg”, “0.2 µg” and “0.5 µg” “1 µg” and “2 µg” represent the sera that was depleted by the corresponding amount of VP30 protein.

Probing 10K random peptide microarray with anti-VP30 antibody depleted serum

Once we had validated the VP30 protein and tested our depletion strategy, we began preparing depleted sera for probing experiments against a peptide microarray. We depleted post-immunization sera with the control protein and two different amounts of VP30 protein separately. Also, the pre-immunization sera were depleted by the control protein and

VP30 protein separately. By comparing the peptide expression profiles of depleted and nondepleted post-immunization sera, we hoped to sort out a set of peptides that represent anti-VP30 antibody activity. The logic behind this was that once the anti-VP30 antibody was depleted, the peptides that had bound to the antibody should no longer light up on the microarray. However, we were not able to find such peptides by t test. In fact, none of the peptides had significant changes in expression level after depletion (p value≤1e-4). We rationalized that the anti-VP30 antibody may not be completely depleted for two reasons. Firstly, VP30 was phosphorylated during the post translational modification (Prados, Vinuela et al. 1993; Hernaez, Escribano et al. 2008). Antibody elicited by phosphorylated VP30 may not be recognized by native VP30. Secondly, during infection, VP30 interact with other proteins such as ribonucleoprotein K (RNP-K) (Hernaez, Escribano et al. 2008). Thus, antibody induced by these conjugated VP30 may also be unrecognized by native VP30 we made by IVTT.

Elution of anti-VP30 antibodies from IVTT beads

Since the depletion experiment could not identify the peptides that had bound to the anti-VP30 antibodies, we changed our strategy. We began by recovering the antibodies bound to the VP30 protein by eluting with an acidic glycine hydrochloride (glycine-HCl) buffer. We eluted the VP30 protein beads three times with a buffer of pH3.8 and then another three times with a buffer of pH5.0. As can be seen in the silver stained SDS-

PAGE gel in Fig. 17, the majority of the antibodies were released in the first elution. The two fat bands are likely to be the full length antibody (the upper band) and the heavy chain of the antibody (the lower band). After the sixth elution, the remaining beads were boiled so every possible protein left on the beads could be collected. Although it is very dim, the band of the VP30 protein is still visible (see the arrow).

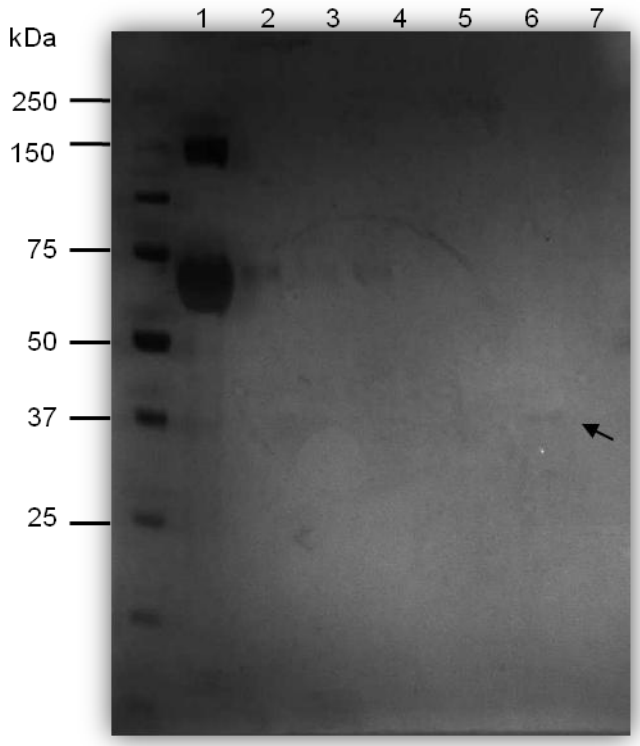


Figure 17. Silver-stained SDS-PAGE gel of purified Anti-VP30 antibody. Lane 1: Flow through from the first elution with pH=5.0 elution buffer. Lane 2: Flow through from the second elution with pH=5.0 elution buffer. 3. Flow through from the third elution with pH=5.0 elution buffer. 4. Flow through from the fourth elution with pH=3.8 elution buffer. 5. Flow through from the fifth elution with pH=3.8 elution buffer. 6. Flow through from the sixth

elution with pH=3.8 elution buffer. 7. Leftover proteins on the beads collected from boiling in 4x loading dye with 20% BME

Probing 10K random peptide microarray with eluted anti-VP30 antibody

After we recovered the anti-VP30 antibodies, we probed for these antibodies on a microarray. We then compared the peptide reactivates with those of unimmunized sera. The five peptides that passed the t test are:

(1) WYQLDAHESYINNLVFP

(2) TMETAKQKTYNILIWYY

(3) AQYMLKLIHYHLIAFQG

(4) SHYNHTIYLRIKKPNAY

(5) WWVITAGWWGIAKIEAG

Using the heat map display, we can see that these peptides show the highest expression level in the retrieved antibody group, the lowest expression in the pre-immunization group, and medium expression in the post-immunization group. Such trends also meet our expectations.

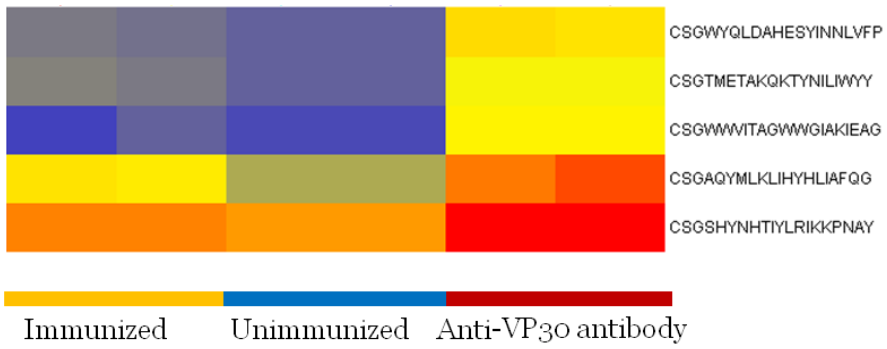


Figure 18. Heat map clustered by 5 selected peptides

The binding of monospecific antibodies to antigens can be described by the “lock and key” model: these antibodies bind only to antigens with a specific epitope. Thus it was predicted that there would be a common motif among the peptides that bound to the anti-VP30 antibody. When the sequence of the first 4 peptides we selected earlier was analyzed by the motif-finding program GLAM2, the motif SHYNNLIF was found (peptide 5 was excluded since it is very different from the other four and contains many tryptophans. This amino acid is considered a sticky residue that may enhance nonspecific binding of antibody). Though not very strong a motif, it was found repeatedly over ten runs. Random peptides analyzed the same way were found to have similarly long motifs without such good consistency or scores.

Later, we included the original protein sequence of VP30 and aligned it with the 4 peptides. The best motif we found was the same as the one we found earlier. The motif was also found repeatedly over multiple runs.

We then tried to find the antibody epitopes for VP30 to see if it matched the motif we found. Of the 4 major epitopes predicted by the IEDB antibody epitope prediction program (Fig. 19), none matched the motif we found. However, the lack of matches does not necessary mean our finding is wrong since the IEDB epitope prediction is based on its own algorithm that is not always very accurate. Also, IEDB searches only for linear epitopes and is unable to find conformational epitopes.

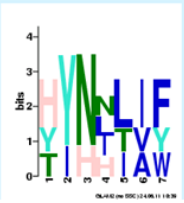
(A)

Best Motif Found:

NAME	START	SITES	END	STRAND	MARGINAL SCORE
1	10	Y I N N L V F	16	+	4.31
2	9	T Y N I L I W	15	+	8.19
3	9	H Y N L I A F	15	+	6.48
4	2	H Y N N T I Y	8	+	9.74

Score: **18.7439**

logo with ssc



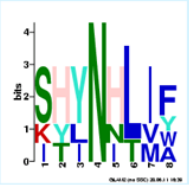
(B)

Best Motif Found:

NAME	START	SITES	END	STRAND	MARGINAL SCORE
peptide1	9	S Y I N N L V F	16	+	7.30
peptide2	8	K T Y N I L I W	15	+	4.14
peptide3	8	I N Y . N L I A	14	+	6.99
peptide4	1	S N Y N N T I Y	8	+	14.9
vp30	198	S N L . N L N F	204	+	9.34

Score: **25.0143**

logo with ssc



(C)

No.	Start Position	End Position	Peptide
1	20	21	RS
2	69	69	Y
3	71	81	GQGYTEHQAQE
4	94	117	ETESSASSENIHEKNDNETNECTS
5	124	134	EQEPSSEVPKD
6	150	157	QYGKAPDF
7	173	180	TPLKEEEK

(D)

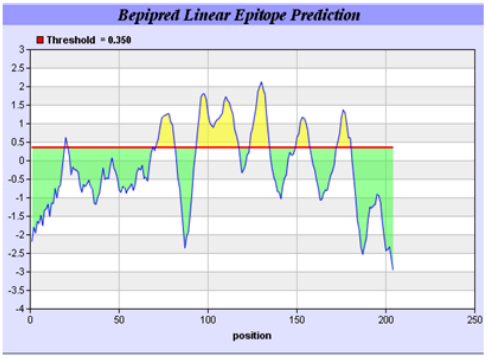


Figure 19. Analysis of motif alignment and VP30 epitope prediction. (A) Best motif found among 4 high-binding peptides. (B) Best motif found among four high-binding peptides and VP30 protein. (C) Predicted linear epitopes by Bepipread prediction. (D) Bepipred linear epitope prediction score.

Chapter 6

DISCUSSION

We have investigated the antibody responses of different ASFV infections and antigen immunizations with a new microarray platform that features 9,786 random-sequence peptides. By probing this microarray with infected and immunized sera, first, we confirmed the hypothesis that antibody responses against ASFV antigen library immunizations and infections could be detected on the random peptide array.

Second, we identified a set of 50 peptides that could distinguish antigen immunized from unimmunized sera with 95% accuracy. Of the four groups of antigen immunized sera, group 4 was most different from the other three groups, which may indicate that the additional 20 randomly selected antigens can influence the immune responses elicited from VP30 and VP72.

Third, we discovered that ASFV infected French and British sera can be distinguished from uninfected sera with 100% accuracy. Although the sample size was small, the clear separation of infected and uninfected sera on the PCA plot demonstrates the microarray's potential for diagnostics.

Fourth, we tested if unexposed sera could be distinguished from “exposed” sera - that is, either infected or library antigen immunized sera. We found that while the separation between the groups was insignificant on PCA, the accuracy was still acceptable (about 85%). The exposed sera

were much more scattered on the plot than the unexposed sera, which may indicate a wide diversity of immune responses among the immune stimulated pigs. We also observed that unlike infected sera, the different groups of the immunized sera do not seem to be segregated by the same PCA analysis. Considering that all the immunizations were boosted with a ASFV antigen-carrying, recombinant vaccinia virus vector, we reasoned that the vaccinia may strongly influence antibody reactivities against random peptides. Many literatures have demonstrated that a minority of vaccinees boosted with vaccinia virus are mainly responded to the vaccinia virus (Gallimore, Cranage et al. 1995; Goulder, Phillips et al. 1997; Hanke, Samuel et al. 1999), which indicate that the antibody reactivities we detected on peptide array may mainly reflect the immno-responses against vaccinia. On the other hand, there are many similarities between vaccinia and ASFV (Afonso, Alcaraz et al. 1992). The ability of our peptide microarray to distinguish ASFV infections from a very similar viral infection also underscores its potential as an ASF diagnostic.

Finally, by probing the random peptide microarray with the purified anti-VP30 antibody, we found a unique set of peptides that bind to anti-VP30 antibody. Using motif finding and alignment approaches, we found a common motif among the peptides and the original VP30 protein sequence. This may indicate that even though the random peptides on our microarray have no homology to the VP30 protein, the binding signals we

detected are not nonspecific responses. Also, it holds the potential of using the random peptide microarray to predict the immunogenic antigens.

In sum, this study not only showed the feasibility of CIM's 10K random peptide microarray as a powerful platform for assessing antibody responses to viral infections and viral antigen immunizations, and also confirmed that pig serum antibody repertories possess sufficiently rich source of immune information for highly useful diagnostic discovery. To our knowledge, this is the first study that applies this platform to assess a pig disease. The peptides we selected to distinguish ASFV antigen-immunized pigs from unimmunized pigs should be carefully used since the selection were probably influenced by the vaccinia-boost. The peptides selected to distinguish infected pigs from uninfected pigs may be considered as the candidates of ASF diagnostic peptides. Given the small sample size, our experiment can only be regarded as a preliminary study. More ASFV-infected samples are required for the validation and the further selections. Nevertheless, the obviously distinct pattern of antibody profiles from exposed pigs confirmed a strong correlation between the antibody responses to ASFV infection, which could lead us to a successful path of diagnosis.

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