RESEARCHREPORT
SWINE HEALTH

Title: Gene expression in lymph nodes of PRRSV-infected pigs – **NPB #08-247**

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iIdustry Summary
The aim of this study was to acquire a better understanding of PRRS disease through a deeper knowledge of gene expression changes that occur in pulmonary lymph nodes during acute comparative porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and swine influenza virus (SIV) infections. The PRRSV, SIV and PCV-2 viral infections followed a clinical course in these domestic pigs typical of experimental infection of young pigs with these viruses. PRRSV isolate SDSU-73 was pathogenic in this study inducing fever, anorexia, listlessness, and dyspnea. Differentially expressed tags (with respect to control) at all time points were ascertained. The experimental results were integrated with previous studies to develop a robust model of swine respiratory virus infection.

Keywords
PRRSV, PCV2, SIV, lymph node, gene expression, functional genomics

Scientific Abstract
Porcine reproductive and respiratory syndrome virus (PRRSV) is a major pathogen of swine worldwide and causes considerable economic loss. Previous in vitro PRRSV infection studies of the primary target cells, porcine alveolar macrophages (PAMs) in our lab have identified,through Serial Analysis of Gene Expression (SAGE) data, specific pathways that associate with variation in PRRSV replication and macrophage function. The goal of the current study to identify significant changes in gene expression in homogenized tracheobronchial lymph nodes (TBLN) during the acute phase of a PRRSV infection in vivo using Digital Gene Expression Tag Profiling. Comparative functional genomics of the acute response against PRRSV, porcine circovirus type 2 (PCV2), and swine influenza virus (SIV) will more clearly define the negative effect of PRRSV on the pig immune system. 12 sows purchased from a source that can provide PCV2, SIV, and PRRSV negative animals were farrowed at NADC to provide 80 early-weaned pigs for the animal experiment at 5 weeks of age. Pigs were allotted to one of 4 equal sized treatment groups according to litter PCV2 maternal antibody status: Group 1 – sham inoculated control, Group 2 – PRRSV challenge, Group 3 - PCV2 challenge, or Group 4 SIV-challenge. On 0 dpi pigs received an intranasal challenge with 2 ml of either sham or virus inoculum. Challenge viruses were PRRSV SDSU73, PCV2 Group 2 European-like, and SIV H1N1 OH07 given at 1 x 10^5 cell culture infectious dose 50% ($CCID_{50}$) per pig. Sham inoculum was prepared from the 3 cell cultures (MARC-145, PK-15, and MDCK cells) used to propagate the viruses. Five pigs from each group were euthanized and necropsied on 1, 3, 6, and 14 dpi. Temperatures of pigs intended for necropsy on 14 dpi were recorded daily. As expected, each virus had its own unique febrile response signature. Pigs in the PRRSV group exhibited dyspnea and lethargy beginning at 8 dpi. At necropsy, lungs were scored for

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gross lesions. Bronchioalveolar lavage fluid (BALF) was cultured for presence of bacterial pathogens. Sections of tracheal-bronchial lymph nodes (TBLN) were homogenized and sent for flow cytometry analysis, cytokine analysis or RNA extraction. Total RNA were collected from TBLN, pooled for each group and timepoint to make 16 libraries, for analysis with Digital Gene Expression Tag Profiling whole-genome expression analysis platform (Illumina Technologies). The data generated underwent image analysis, base calling, and standard filtering to generate a list of sequence tags and counts. Multidimensional statistical tests and clustering analysis were applied to determine which changes in tag abundance are significant. Tags were annotated with available transcript information. The updated analysis pipeline contains 7804 swine RefSeq sequences and 240420 HarvardGI Accessions (SSGI release 14) allowing us to associate tags with transcripts and genes. Virus sequences from Refseq and GenBank allowed us to determine viral tag counts in the libraries. The experimental results have been integrated with previous studies to develop a robust model of swine respiratory virus infection. For select genes of interest, significant changes were validated by real-time RT-PCR, and changes in transcript abundance mapped to known metabolic, signaling and other pathways/networks. Gaining insight into how the virus causes disease may aid development of more cross-protective vaccines that would certainly lead to the production of healthier swine. If more efficacious vaccines were available, then they may lead to strategies to eliminate PRRSV from U.S. swine, a feat that would provide long-term economic impact.

Introduction

This investigation is an in vivo comparative study into the swine host immune response to respiratory infection using a new technology capable of detecting global changes in gene expression. Respiratory diseases are extremely costly to the swine industry worldwide and ongoing research is essential for gaining a better understanding of the pathogenesis, diagnosis, and prevention of respiratory disease. The lymph node is the place where the innate (early, non-specific) immune response talks to the adaptive (later, specific) immune system. Tracheobronchial lymph nodes (TBLN) drain the virus-infected tissues (inflamed lungs). The advantage of sampling the TBLN is that we are studying both direct effects of virus on cells in the lymph nodes as well as indirect effects on lymph nodes draining the lungs and therefore are studying at least a portion of the real host response. The disadvantage is that the results are the combined efforts of a number of cell types, which may or may not have responded to viral infection. Gene expression microarrays are at present the default technology for transcriptome analysis. Since they rely on sequence-specific probe hybridization, they suffer from background and cross-hybridization problems and measure only the relative abundances of transcripts. Also, because the pig whole genome assembly is not yet achieved, no complete pan-genomic array exists and only partial generic microarrays are commercially available. In contrast, tag-based sequencing methods like SAGE (Serial Analysis of Gene Expression) measure absolute abundance and are not limited by array content. Digital Gene expression Tag profiling (DGETP) is the most advanced derivate of the SAGE technology for the analysis of expressed genes in eukaryotic organisms. Like in SAGE, a specific tag from each transcribed gene is recovered. By sequencing and counting as many tags as possible, the transcription profile, stating what gene is described and how often, becomes apparent. DGETP uses the restriction enzyme, *DpnII*, to cut 21 bp long sequence tags from each transcript's cDNA. The longer tag-size allows for a more precise allocation of the tag to the corresponding transcript, because each additional base increases the confidence in the mapping of the tag to a transcript or genomic position. We used the Solexa/Illumina Genome Analyzer II, in which adapter sequences, ligated to both ends of the DNA molecule, are bound to a glass surface coated with complementary oligonucleotides. This is followed by solid-phase DNA amplification and sequencing-by-synthesis . The system yields millions of short reads (currently up to 36 bp), and is therefore very suitable for tag-based transcriptome sequencing. Each transcript can be quantified by counting the tags in a DGETP library such that quantitative genetics is possible. The proposed animal experiment was expanded to study the acute response against porcine circovirus type 2 (PCV2), and swine influenza virus (SIV). Although PRRSV, PCV2, and SIV can induce respiratory disease in pigs that may appear similar, the mechanism for each virus is probably different reflecting their unique properties. PCV2 like PRRSV can directly effect cells within lymph nodes as well as cause inflammation in lungs. We do not anticipate any direct effect of SIV on cells within lymph nodes, but they will be affected by the inflammation or pneumonia. Understanding the host response by studying gene expression may lead to the development of methods that shorten or prevent a chronic PRRSV infection as well as improve cross-protection of PRRSV vaccines; achievements that would be key components of any successful vaccinate-to-eradicate program. An expanded study that allows the comparison of PRRVS, PCV2, and SIV host responses will provide greater insight into the basic immune response of swine to respiratory disease.

Objectives

The objective of this research project is to identify significant changes in gene expression during the acute phase of a PRRSV infection. In addition, if funding permits, the proposed animal experiment will be expanded to study the acute response against porcine circovirus type 2 (PCV2), and swine influenza virus (SIV).

Aim 1. 80 weaned pigs will be randomly allotted to one of 4 equal treatment groups: Group 1 sham inoculated control, Group 2 PRRSV challenge, Group 3 PCV2 challenge, or Group 4 SIV-challenge. On 0 days post-infection (dpi) pigs will receive an intranasal challenge 1 x 10^5 cell culture infectious dose 50% ($CCID_{50}$) per pig according to their assigned group. Five pigs from each group will be euthanized and necropsied on 1, 3, 6, and 14 dpi. At necropsy, lungs will be scored for gross lesions. Bronchioalveolar lavage fluid (BALF) and tracheal-bronchial lymph nodes (TBLN) will be collected. Sections of TBLN and lung will be placed into formalin for histopathology. All 0, 1, 3, 6, and 14 dpi sera, and BALF will be tested for respective virus.

Aim 2. Total RNA will be collected from TBLN, pooled for each group and time point to make 16 libraries, for analysis with Digital Gene Expression Tag Profiling whole-genome expression analysis platform (Illumina Technologies). The data generated will undergo image analysis, base calling, and standard filtering to generate a list of sequence tags and counts.

Aim 3. Multidimensional statistical tests will be applied to determine which changes in tag abundance are significant. Tags will be annotated with genomic information and differential gene expression analyzed. The experimental results will be integrated with previous studies to develop a robust model of swine respiratory virus infection.

Aim 4. For select genes of interest, significant changes will be validated by real-time RT-PCR, and changes in transcript abundance will be mapped to known metabolic, signaling and other pathways/networks.

Materials and Methods

Aim 1. 12 sows purchased from a source that can provide PCV2, SIV, and PRRSV negative animals were farrowed at NADC to provide 80 early-weaned pigs for the animal experiment at 5 weeks of age. Pigs were allotted to one of 4 equal sized treatment groups according to litter PCV2 maternal antibody status: Group 1 – sham inoculated control, Group 2 – PRRSV challenge, Group 3 - PCV2 challenge, or Group 4 SIV-challenge. Each group was housed in an isolation room for about one week prior to the beginning of the experiment. Blood samples were collected on 0, 1, 3, 6, and 14 days post-inoculation (dpi). Temperatures of pigs intended for necropsy on 14 dpi were recorded daily. Pig weights were recorded on 0 dpi and at necropsy. On 0 dpi pigs received an intranasal challenge with 2 ml of either sham or virus inoculum. Challenge viruses were PRRSV SDSU73, PCV2 Group 2 European-like, and SIV H1N1 OH07, used previously in our laboratory and given at a similar dose, about 1 x 10^5 cell culture infectious dose 50% ($CCID_{50}$) per pig (Lager et al. 1996; Lager et al., 2007, Vincent et al., 2007). Sham inoculum was prepared from the 3 cell cultures (MARC-145, PK-15, and MDCK cells) used to propagate the viruses. Five pigs from each group were euthanized and necropsied on 1, 3, 6, and 14 dpi. At necropsy, lungs were scored for gross lesions. Bronchioalveolar lavage fluid (BALF) and tracheal-bronchial lymph nodes (TBLN) were collected. 2ml of BALF was frozen at -80°C. BALF was cultured for presence of bacterial pathogens. A section of TBLN was homogenized and sent for flow cytometry analysis. Another section of TBLN was homogenized in tissue lysis buffer for cytokine analysis. Remaining TBLN was stored in RNAlater at -80°C for RNA extraction. Sections of lung and thymus were placed into formalin for histopathology. In each treatment group all 0 and 14 dpi sera was tested for respective antibody. All 0, 1, 3, 6, and 14 dpi sera, and BALF were tested for respective virus. Testing for virus included virus isolation on cell culture and/or quantitative PCR. The in vitro assays described above are routinely performed in our laboratory (Lager et al. 1996; Lager et al., 2007, Vincent et al., 2007).

Aim 2. Total RNA was collected from TBLN, pooled for each group and time point to make 16 libraries, for analysis with Digital Gene Expression Tag Profiling whole-genome expression analysis platform (Illumina Technologies) (submitted August 13th, 2009). The data generated underwent image analysis, base calling, and standard filtering to generate a list of sequence tags and counts (completed for all libraries February 11th, 2010).

Aim 3. Multidimensional statistical analysis is underway in order to determine which changes in gene expression based on tag abundance are significant. Tags are being annotated with available transcript information.

Aim 4. For select immune response genes cytokine ELISAs (IFN-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α) and real-time RT-PCR (β-actin, IFN-α, IFN-β, Mx1, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, TNF-α, CCL2, CCL5) have been completed.

Results

Aim 1. Animal study

Figure 1A. Pig rectal temperatures

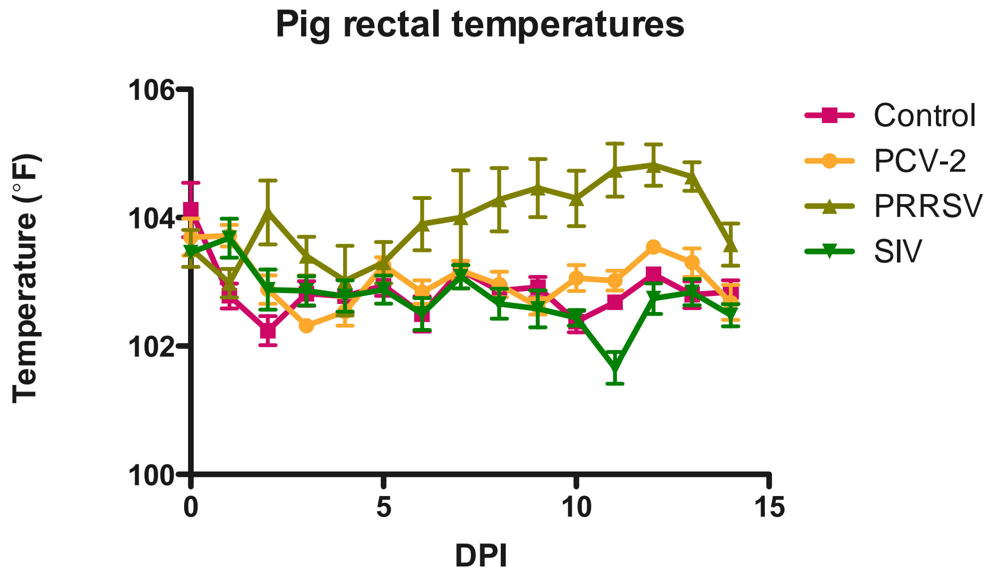


Figure 1B. Pig weights

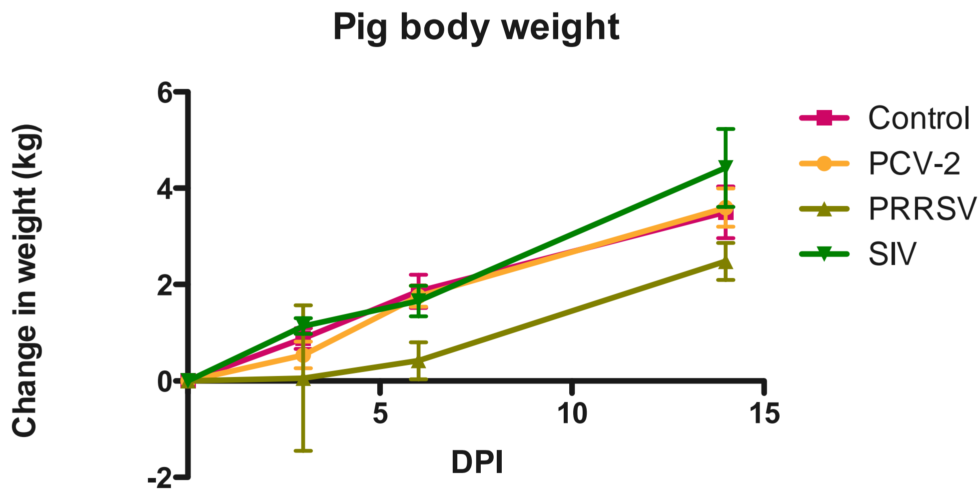
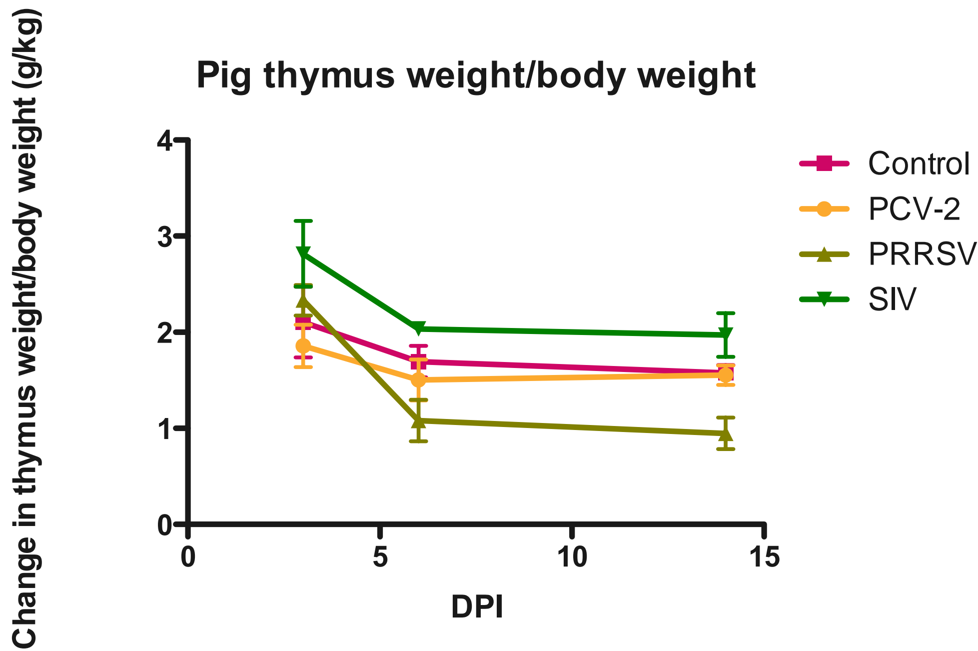


Figure 1C. Pig thymus weights



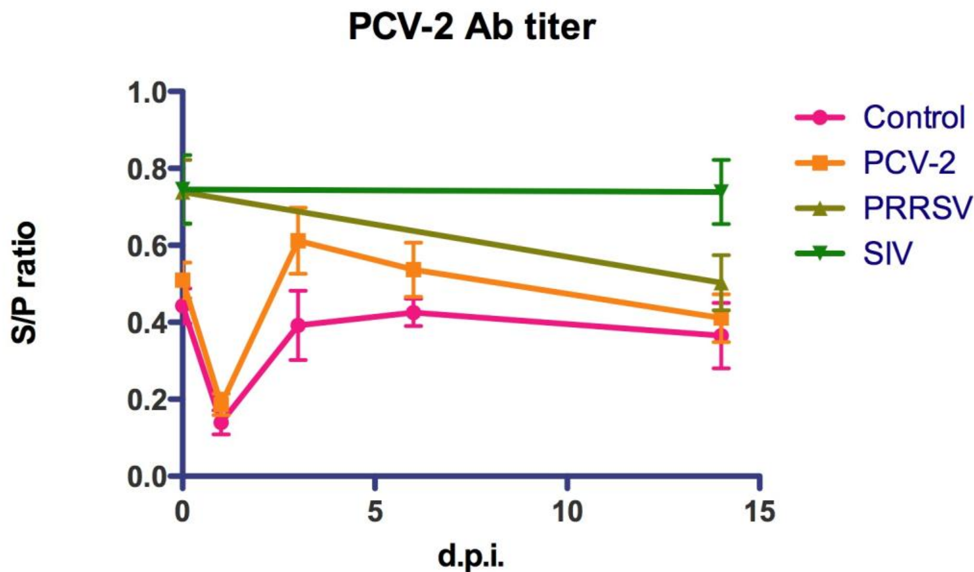
Clinical evaluation. The elevation of rectal temperature during the experiment is shown in Fig. 1A. As expected, each virus had its own unique febrile response signature. Pigs inoculated with PRRSV had a biphasic increase in rectal temperature with an initial peak at 2 dpi and a second sustained increase between 6 to 14 dpi. Pigs inoculated with PCV-2 had only a slightly increased rectal temperature from 10 to 14 dpi. Pigs inoculated with SIV had a transiently increased rectal temperature from 1 to 2 dpi. Pigs in the PRRSV group exhibited dyspnea and lethargy beginning at 8 dpi. Body weight change during the experiment is shown in Fig. 1B. Average daily weight gain was highest in pigs inoculated with SIV (0.31 kg/day), followed in decreasing order by pigs inoculated with PCV2 (0.26 kg/day), control pigs (0.25 kg/day), and PRRSV (0.18 kg/day).

Table 1. Lung macroscopic lesions scores

	3 d.p.i.	6 d.p.i.	14 d.p.i.
Sham	0.34 ± 0.3	0.14 ± 0.1	0 ± 0
PCV-2	0.11 ± 0.1	1.36 ± 1.2	1.04 ± 0.6
PRRSV	5.27 ± 1.6	8.6 0± 3.4	57.05 ± 7.8
SIV	8.96 ± 1.4	31.48 ± 2.0	1.30 ± 0.5

Gross findings. Macroscopic lesions are summarized in Table 1. The PRRSV-inoculated pigs had lungs with diffuse tan mottling at 14 dpi. The SIV-inoculated pigs had interstitial edema and dark areas on the lung surface at 6 dpi. PCV-2-inoculated pigs and control pigs did not have any macroscopic lesions. No significant bacteria were isolated from the BALF of any of the pigs.

Figure 2. PCV-2 antibody titer



Serological analysis. Antibodies to PRRSV were only detected in PRRSV-inoculated animals; animals in the rest of the groups remained seronegative to PRRSV during the entire experiment. The PRRSV-inoculated pigs seroconverted at 14 dpi. Only pigs inoculated with SIV seroconverted to this virus at 14 dpi; pigs in the rest of the groups remained seronegative to SIV during the experiment. Low levels of maternal antibody to PCV-2 virus was present in all pigs (Figure 2) and there was no evidence of seroconversion by 14 dpi.

Table 2. Detection of virus

Tissue	Treatment group	Virus detected	0 d.p.i.		1 d.p.i.		3 d.p.i.		6 d.p.i.		14 d.p.i.	
			RT-PCR	VI	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI
Serum	Control	PCV-2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		PRRSV	0/5		0/5		0/5		0/5		0/5	
		SIV	0/5		0/5		0/5		0/5		0/5	
	PCV-2	PCV-2	0/5	0/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	PRRSV	PRRSV	0/5		5/5		5/5		5/5		5/5	
	SIV	SIV	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
BALF	Control	PCV-2			0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		PRRSV			0/5		0/5		0/5		0/5	
		SIV			0/5		0/5		0/5		0/5	
	PCV-2	PCV-2			4/5	0/5	5/5	0/5	4/5	0/5	4/5	0/5
	PRRSV	PRRSV			0/5	2/5	5/5	2/5	5/5	5/5	5/5	5/5
	SIV	SIV			0/5	3/5	1/5	2/5	1/5	0/5	0/5	0/5
TBLN	Control	PCV-2										
		PRRSV										
		SIV			0/5		0/5		0/5		0/5	
	PCV-2	PCV-2										
	PRRSV	PRRSV			5/5		5/5		5/5		5/5	
	SIV	SIV			0/5		1/5		0/5		0/5	

Quantitative PCR for virus nucleic acid and virus isolation. Table 2 summarizes the virus detection assays completed thus far. In this study the SIV used is from the same genetic cluster as the 2009 novel A/H1N1.

The SIV-inoculated pigs were no longer shedding virus at 14 dpi and all sera tested were negative by real-time RT-PCR and virus isolation at all time points tested.

Aim 2. Digital Gene Expression Tag Profiling whole-genome expression analysis

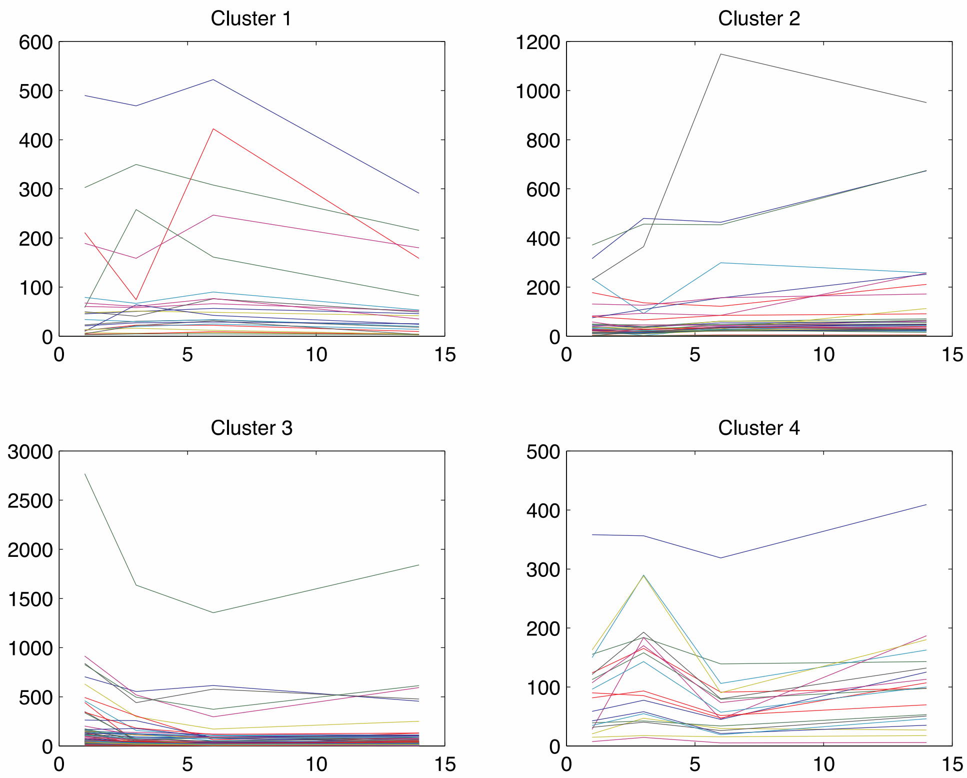
Table 3 Summary statistics from DGE analysis

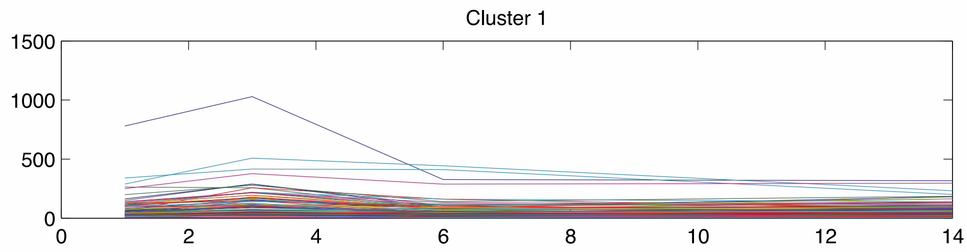
Treatment	dpi	Total raw tag count	Total TPM	Number of unique tag sequences	Total TPM >=2 TPM
Control	1	11,674,409	1,004,559	536,549	906,746
Control	3	11,228,125	1,002,752	448,474	912,566
Control	6	11,327,590	1,002,881	425,634	923,563
Control	14	11,988,170	1,002,911	524,447	913,172
PCV2	1	12,520,070	1,001,711	509,594	916,203
PCV2	3	11,877,759	1,001,729	500,483	915,671
PCV2	6	12,764,159	1,003,530	632,973	894,770
PCV2	14	13,851,757	994,017	560,072	901,886
PRRSV	1	11,277,990	1,001,407	298,655	921,684
PRRSV	3	12,825,498	1,001,241	310,915	951,804
PRRSV	6	9,938,630	998,950	240,000	914,345
PRRSV	14	13,446,606	996,540	290,815	944,725
SIV	1	12,721,121	1,002,342	467,564	923,243
SIV	3	10,304,054	1,002,230	303,170	923,823
SIV	6	15,797,499	998,092	428,811	897,435
SIV	14	12,628,437	1,001,273	371,926	923,877
Total		196,171,874	16,016,165	6,850,082	14,685,513

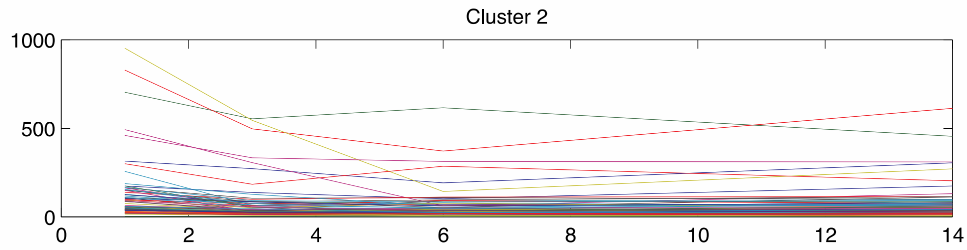
Digital Gene Expression (DGE) analysis. For an in-depth assessment of the respiratory virus-infected pig transcriptome, we generated and analyzed 16 DGE libraries with an average of 12,260,742 tags per library. Linkers, repetitive sequences and transcript tags with a count of < 2 tags per million (TPM) were removed resulting in a total of 14,685,513 transcript tags per million (TPM) sequenced of which 6,850,082 were unique. Table 3 summarizes the tag counts from each library. PRRSV infection reduced the unique tag sequences (i.e., transcriptome diversity) in the TBLN transcriptome at 1, 3, 6 and 14 dpi to 55.7%, 69.3%, 56.4% and 55.5% of control TBLN. PCV2 infection increased the unique tag sequences to 95.0%, 111.6%, 148.7% and 106.8% of controls where as, SIV induced a more modest reduction in unique tag sequences at 87.1%, 67.6%, 100.7% and 70.9% of the respective control transcriptome at 1, 3, 6 and 14 dpi.

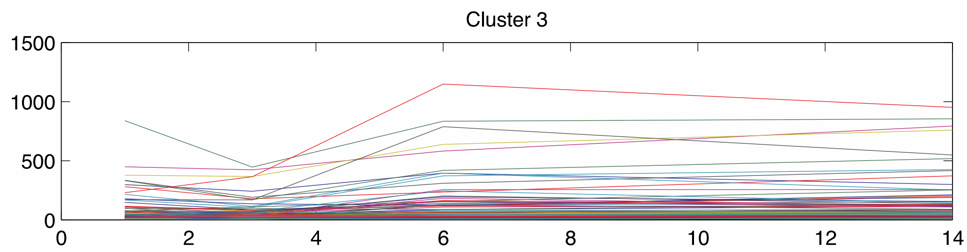
Aim 3. Genomic annotation and differential gene expression analysis

Figure 3. Cluster analysis

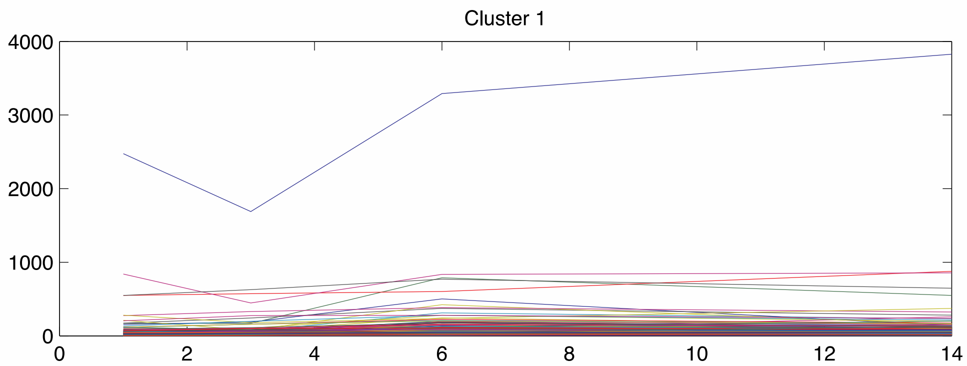


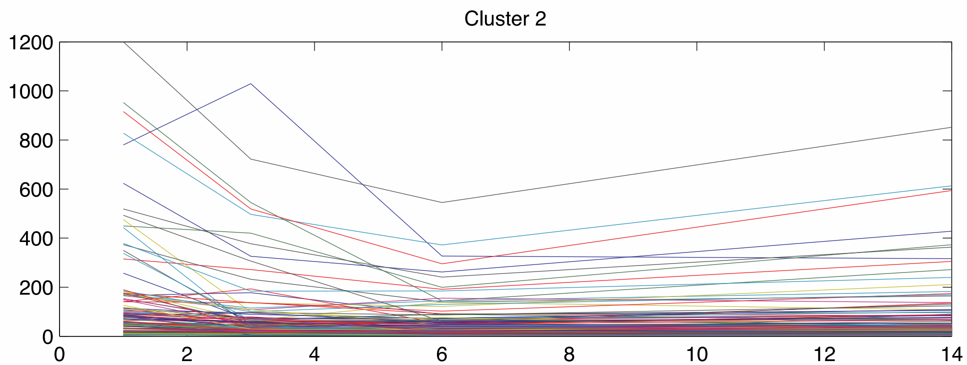




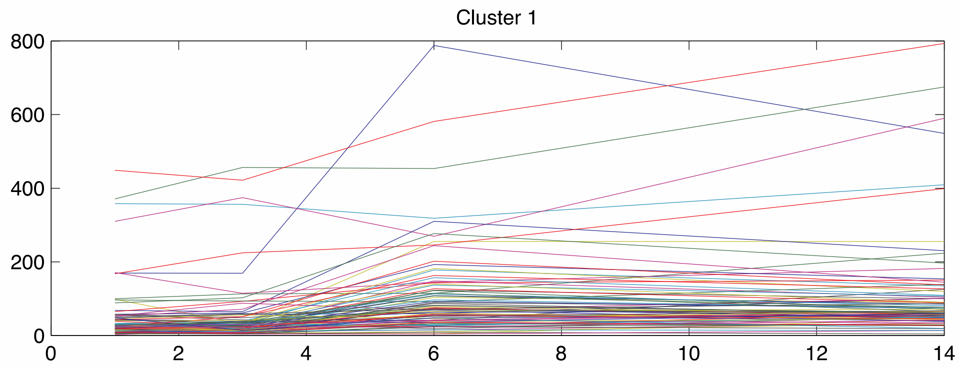


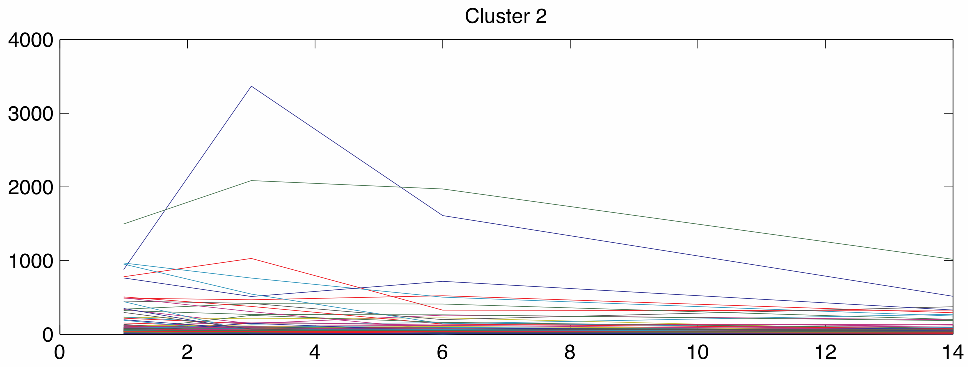
PCV2 - Day 3 - 271 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)



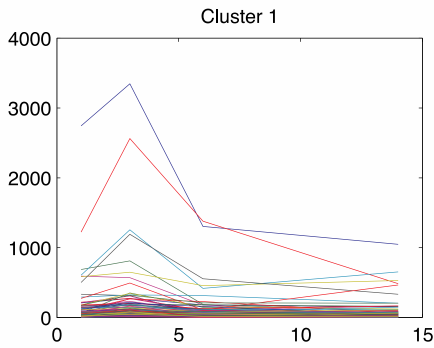


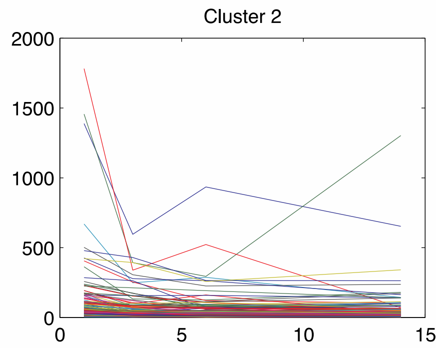
PCV2 - Day 6 - 288 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

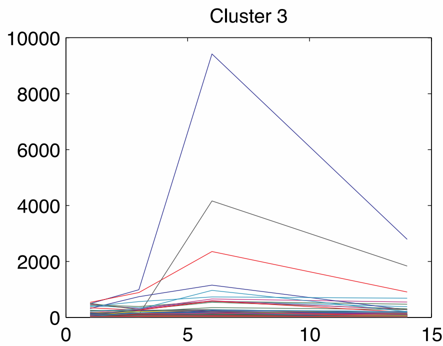


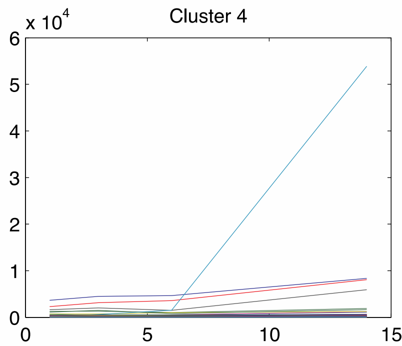


PCV2 - Day 14 - 176 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

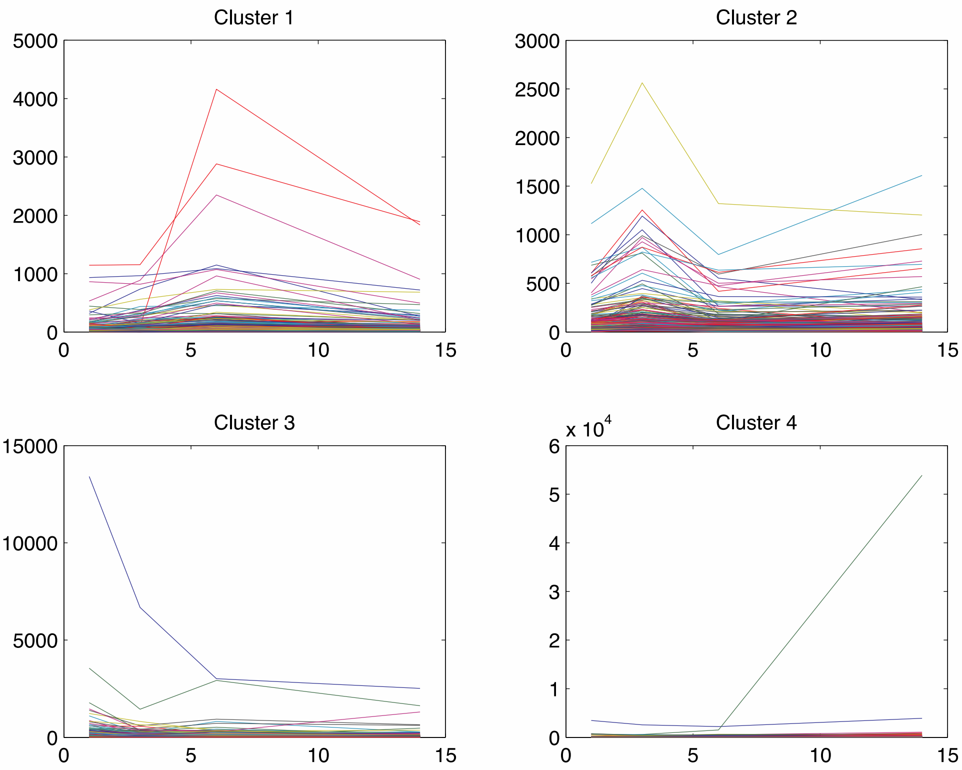




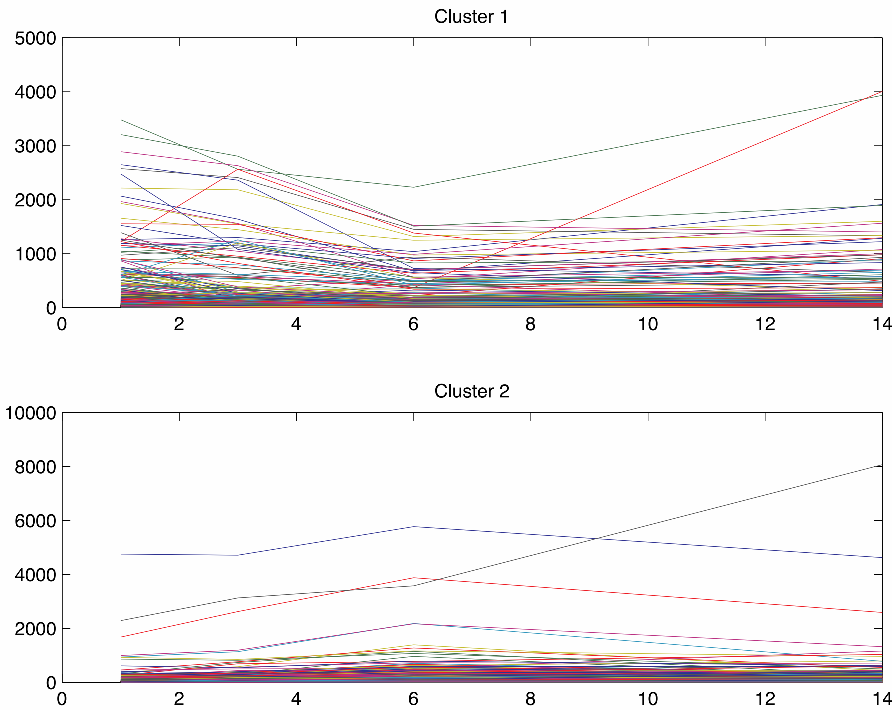




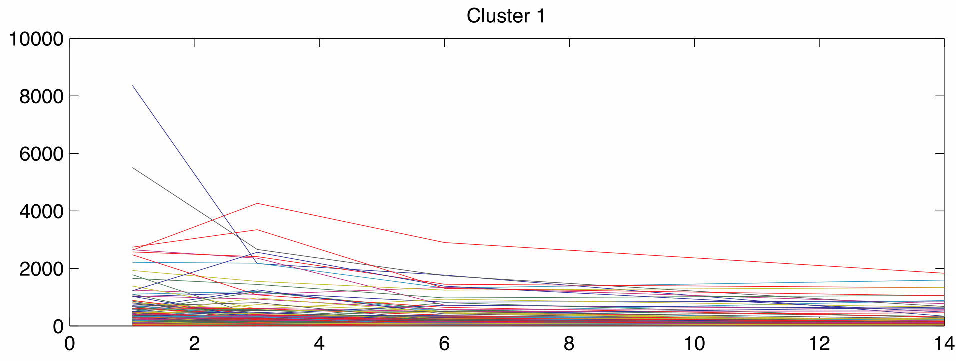
PRRSV - Day 1 - 893 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

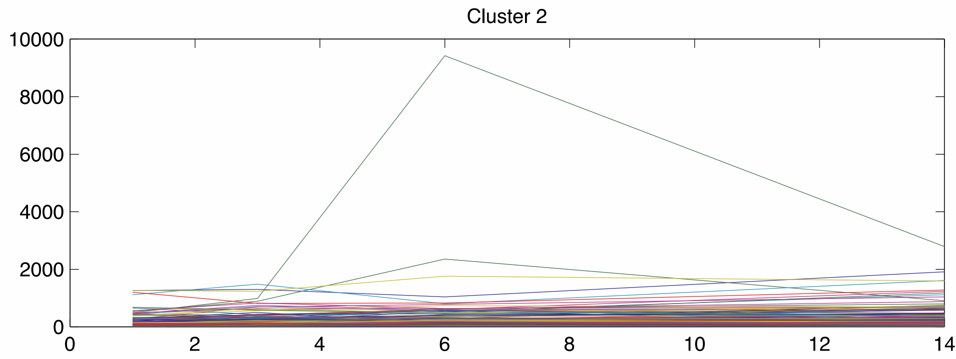


PRRSV - Day 3 - 2439 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

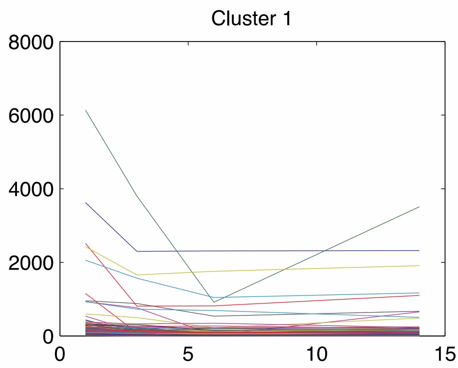


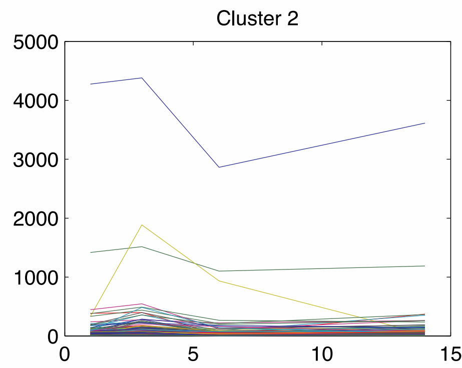
PRRSV - Day 6 - 2472 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

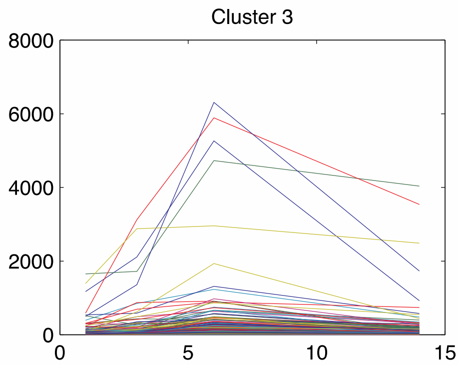


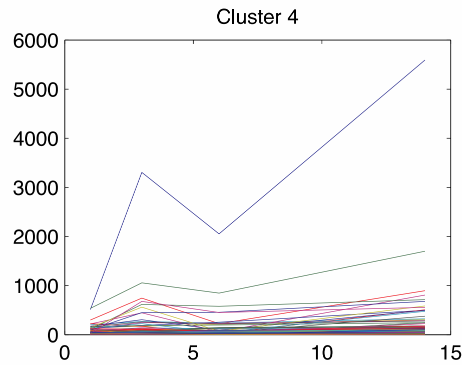


PRRSV - Day 14 - 2287 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

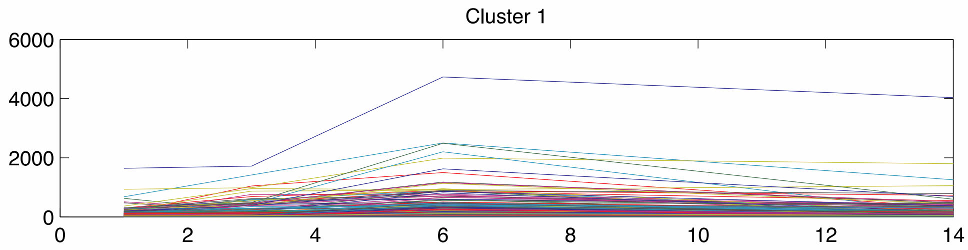


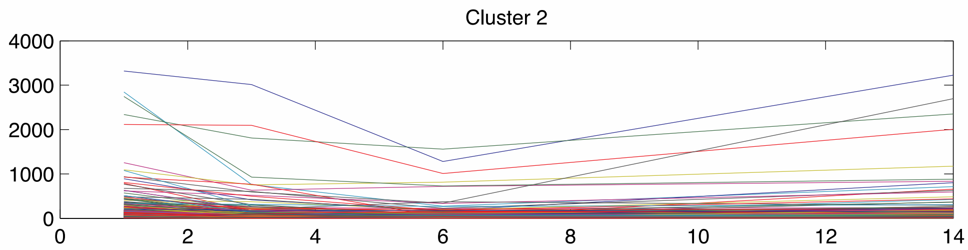


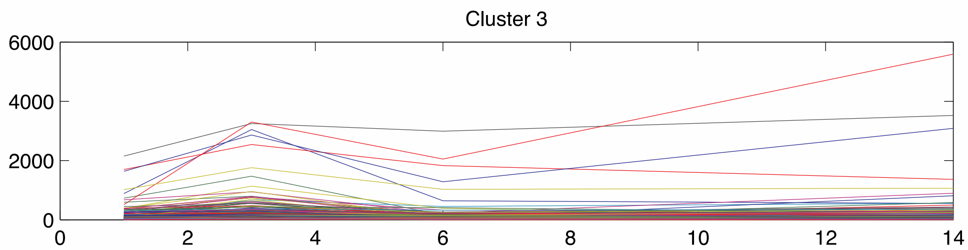




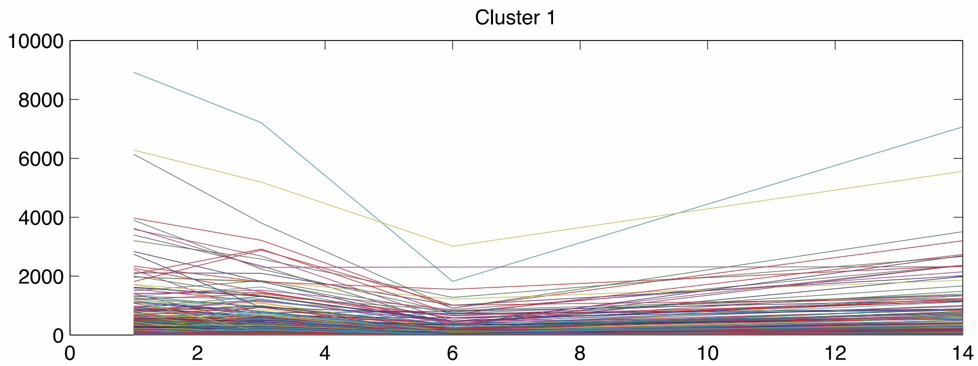
SIV - Day 1 - 733 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

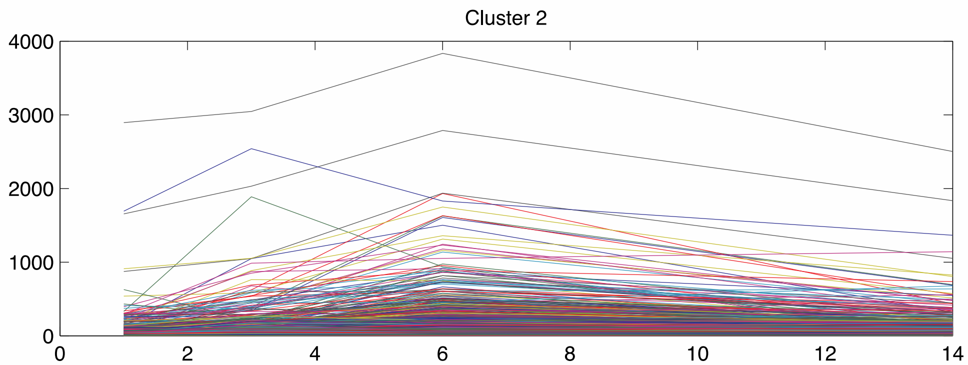




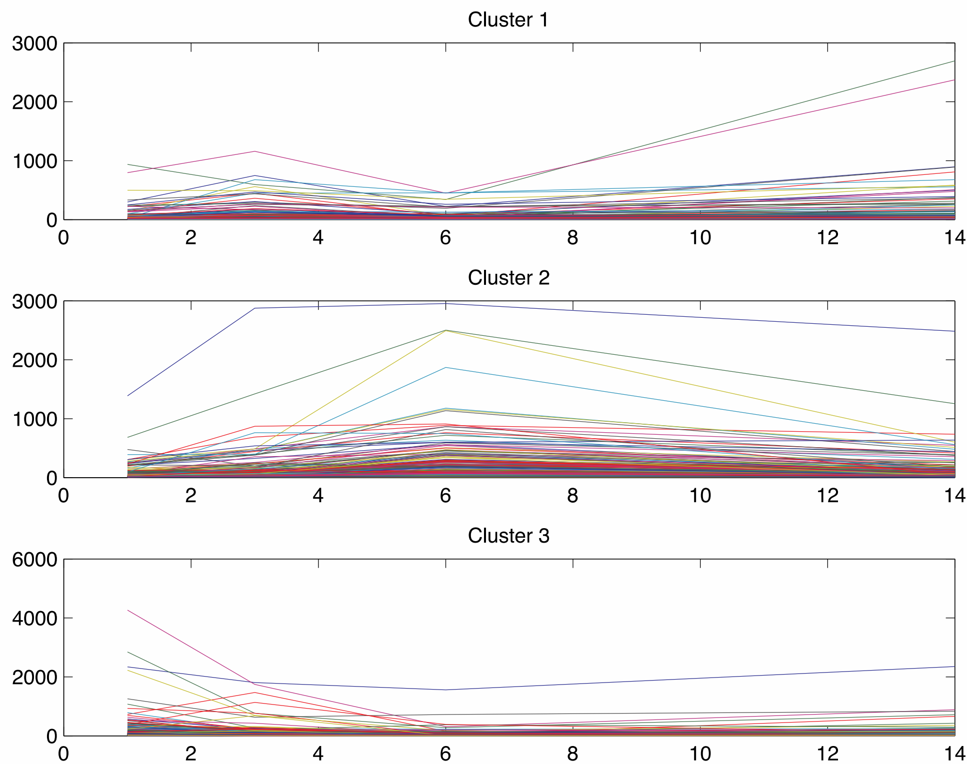


SIV - Day 3 - 1984 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)





SIV - Day 6 - 4540 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)



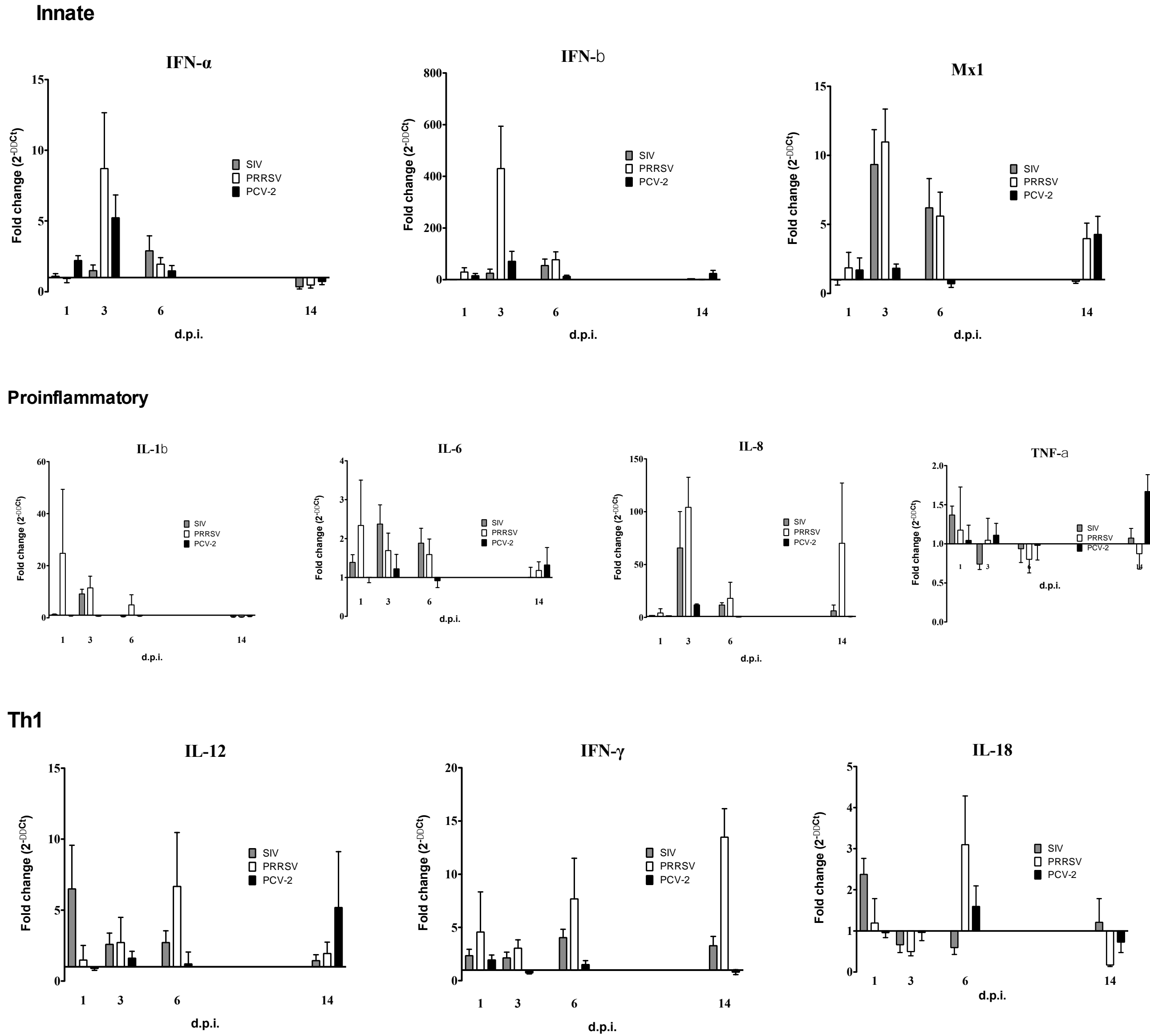
SIV - Day 14 - 1195 Differentially Expressed Tags (Threshold : 2X fold change, q-value <= 0.01)

The behavior of the differentially expressed tags at each time point was followed through out the course of the experiment using Kmeans clustering of tags: cluster with respect to correlation distance (Fig. 3). The number of clusters is selected using silhouette plots, choosing the number of clusters that maximizes the silhouette values for all clusters. It is an objective way of determining the number of clusters. Values allowed were from 2 to 8 clusters in for PCV2, SIV, and PRRSV. Figure 3 shows the optimum number of clusters are different for the different viruses and time points. The number of differentially expressed (DE) tags changes between the different viruses and time points (DE threshold is set a 2x fold change with a false discovery rate (FDR) of q<= 0.01).

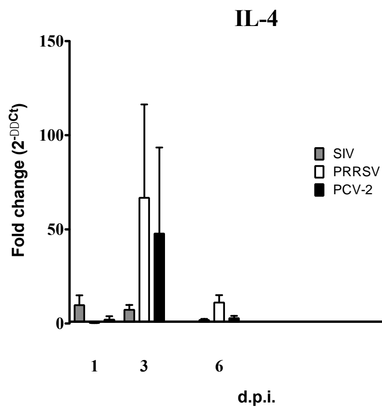
The updated analysis pipeline contains 7804 swine RefSeq sequences and 240420 HarvardGI Accessions (SSGI release 14) allowing us to associate tags with transcripts and genes. Virus sequences from Refseq and GenBank allowed us to determine viral tag counts in the libraries. Tags are currently being associated with genes. This should be completed within the next 2 months.

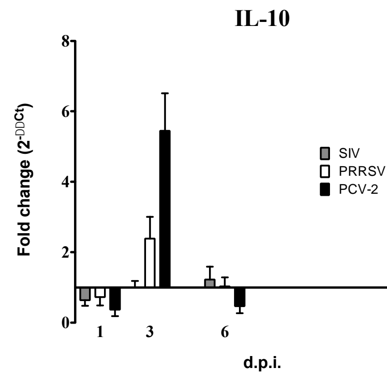
Aim 4. Validation of differential expression by real-time RT-PCR

Figure 4. Significant changes validated by real-time RT-PCR for select genes of interest

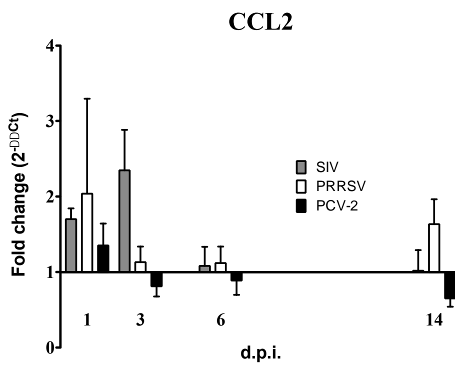


Th2





Chemokines



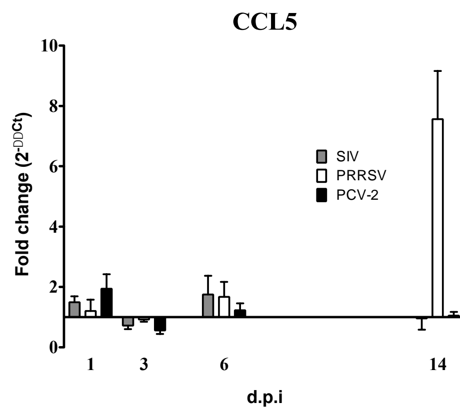
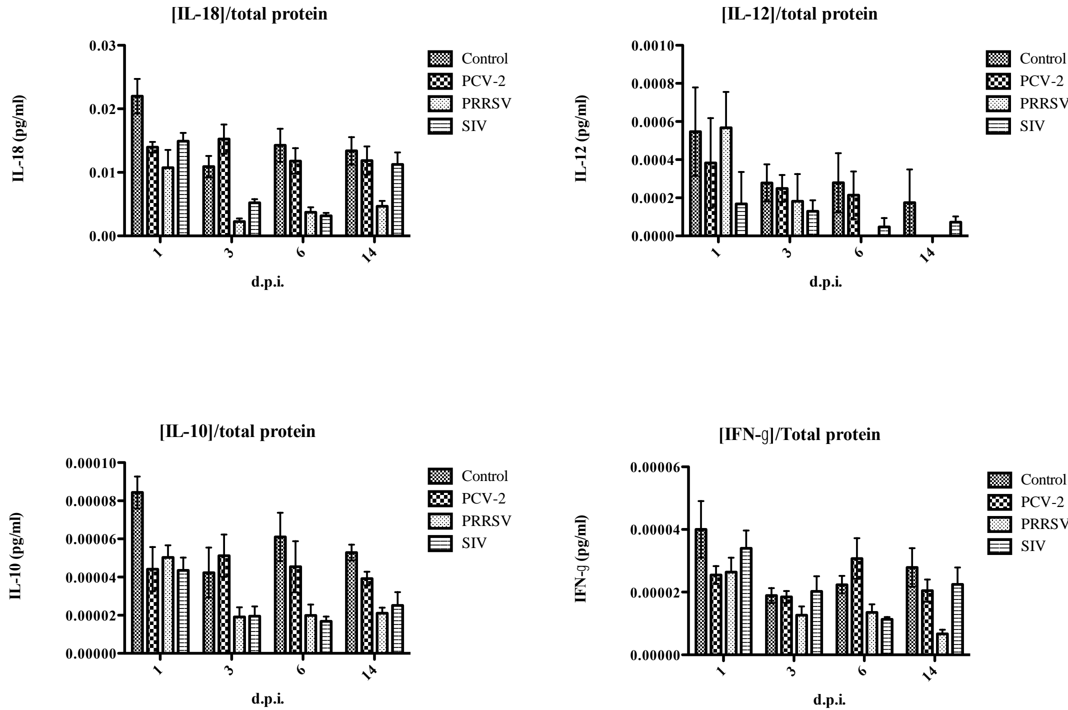


Figure 5. Cytokine concentration in TBLN homogenate of pigs inoculated with virus or controls normalized to total protein concentration per gram of TBLN.



Cytokine protein expression profiles. Figure 4 shows cytokine protein levels in the TBLN post-infection for IL-18, IL-12, IL-10 and IFN-γ measured by ELISA.

Cytokine protein levels in serum and TBLN were analyzed using Searchlight Multiplex Immunoassay array (Aushon Biosystems, Inc.) for IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN-γ and TNF-α. Results are summarized below of the significantly (least square means for between treatment groups) changed cytokine protein levels post-infection.

No effect on serum or TBLN IL-1β.

No effect on serum IL-4 but significant elevation of IL-4 in TBLN in SIV and PRRSV-infected pigs.

No effect on serum IL-6 but significant elevation of IL-6 in TBLN in SIV and PRRSV-infected pigs.

Significant group effect on IL-8 in serum and TBLN, which appears to be suppressed in SIV and PRRSV-infected pigs.

Significant effect on serum and TBLN IL-12p70 levels with most increased caused by PRRSV-infection.

Trend for elevated IFN-γ levels in serum of PRRSV-infected pigs; significant increase in TBLN IFN-γ levels due to PRRSV, less so for SIV.

No effect on serum or TBLN TNF-α.

Differentially expressed genes will be mapped to known metabolic, signaling and other pathways/networks using Gene Set enrichment Analysis (Broad Institute).

Discussion:

The PRRSV, SIV and PCV-2 viral infections followed a clinical course in these domestic pigs typical of experimental infection of young pigs with these viruses. PRRSV isolate SDSU-73 was pathogenic in this study inducing fever, anorexia, listlessness, and dyspnea.

PRRSV infection reduced the unique tag sequences (i.e., transcriptome diversity) in the TBLN transcriptome at 1, 3, 6 and 14 dpi to 55.7%, 69.3%, 56.4% and 55.5% of control TBLN. PCV2 infection increased the unique tag sequences to 95.0%, 111.6%, 148.7% and 106.8% of controls where as, SIV induced a more modest reduction in unique tag sequences at 87.1%, 67.6%, 100.7% and 70.9% of the respective control

transcriptome at 1, 3, 6 and 14 dpi. Differentially expressed tags (with respect to control) at all time point were ascertained and will be mapped to known metabolic, signaling and other pathways/networks using Gene Set enrichment Analysis (Broad Institute). 85% of unique tags identified in this study cannot be associated with curated transcripts (NM_). The findings in significantly differently expressed cytokine transcript abundance were supported in the changes in cytokine protein levels in the serum and TBLN. The picture will likely change dramatically once the annotation improves.

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