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Title: "Year 3 funding support for the PRRS Host Genetics Consortium: A proposal to study the role of host genetics and resistance to PRRSV" – **NPB #10-156**

Investigators: Raymond R. R. Rowland and Joan K. Lunney

Institutions: Kansas State University and USDA ARS BARC

Co-Investigator: James Reecy

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Industry Summary

The PRRS Host Genetics Consortium (PHGC) represents the first-of-its-kind approach to food animal infectious disease research. The NPB, PRRS CAP, USDA ARS, NIFA, Genome Canada, private companies, and universities have come together to conduct a multi-year project to understand how host genetics influences the outcome of PRRSV infection. The thorough characterization of PRRS-associated genomic markers will be used in breeding programs to identify pigs that are more resistant/tolerant to infection, produce a desired antibody or cytokine response, and/or respond well following vaccination (vaccine-ready pigs). Spinoffs from the project include new information and research to improve the control of PRRS in the field, such as oral fluid surveillance for PRRS and other infectious diseases and the characterization of biomarkers linked to specific infection and growth outcomes. Another spinoff is the identification of a pig with severe combined immunodeficiency (SCID), a new model for understanding mechanisms associated with PRRS pathogenesis and immunity.

The principal activities conducted during the latest funding period include the experimental infection of an additional 400 pigs: PHGC7, 8. To date, funding from NPB, USDA, Genome Canada and private companies has supported 14 trials or approximately 2800 pigs. Genomic DNA was prepared from PHGC6, 7, and 8 pigs and their available parents for single-nucleotide polymorphism (SNP) genotyping. Samples (serum, blood RNA Tempus tubes, oral fluids, skin tissue, and tonsils) collected from these trials are stored at K- State and BARC and represent a rich resource available to all PRRS researchers. For every dollar provided by the NPB, the PHGC has contributed more than $10 in matching funds The secure PHGC relational database http://www.animalgenome.org/lunney/index.php continues to be developed for sharing phenotypic and genotypic data, gene and protein expression results, and statistical analyses. Year 3 NPB funding is being used to complete infection of pigs, complete the PRRSV RT-PCR on sera from PHGC 6, 7, and 8 (Rowland), continue building the relational database (Reecy), and support the preparation of genomic DNA and blood cell RNA, and measurement of circulating cytokines (Lunney).

Raymond R. R. Rowland, browland@vet.k-state.edu, 785-532-4631
Joan K. Lunney, joan.lunney@ars.usda.gov, 301-504-9368

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For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Scientific Abstract
The PRRS Host Genetics Consortium (PHGC) is an international effort developed with input from PRRS researchers, NC1037/NRSP8 genome researchers, members of the NPB Swine Health and Animal Science Committees, veterinarians, AASV, producers, and commercial partners. It was initially funded by NPB starting in December 2007. The PHGC incorporates a nursery pig model to assess pig responses to acute PRRSV infection and to study the relationship between host genes and the resistance/susceptibility of pigs to PRRSV infection. Blood and other samples (e.g. oral fluids) and weight measurements are collected regularly. Serum and Tempus tube blood samples are collected at 10 time points for all pigs, providing the opportunity to create "deep phenotypes" of the anti-PRRS response. Tonsil tissue is collected at the end of the study, on day 42. The presence of virus in tonsil is used as a measure of persistent infection. Phenotypic measurements include virus levels, overall virus load, weight gain, antibody responses (total and neutralizing), gene expression and cytokine protein levels in serum. All samples are catalogued and distributed to appropriate testing labs and stored for use in future studies. The data are collected into a secure PHGC relational database, housed at Iowa State University and maintained by James Reecy. Genomic DNA is prepared from each pig and is genotyped using funding from separate PRRS CAP and Genome Alberta grants and through resources provided by the national NRSP-8 swine genome coordinator. RNA is prepared from Tempus blood tubes for Pigoligoarray hybridization studies and total RNA sequence (RNAseq) analysis of host gene expression, which is supported by a separate USDA NIFA grant and recent Genome Canada and Genome Alberta grant funding. Oral fluid samples are collected for the purpose of developing improved PRRS surveillance methods. To date, PHGC activities have produced several important discoveries, including markers on chromosome 1, 4, 7, 17 and X, which are involved in disease resistance and immunity.

Deliverables of the PHGC include:
- Development of genetic and blood tests that can be used to predict how pigs respond to PRRSV infection.
- Determination of alleles in genomic regions, single nucleotide polymorphism (SNP), or candidate genes [and source pig genetics] which are correlated with the response of pigs to PRRSV infection.
- Identification of quantitative trait loci (QTL) to develop selection procedures to lower the effects of PRRS and prevent persistence of PRRSV virus in pigs.
- Discovery of unique PRRSV resistance mechanisms and virus-host interactions.
- Characterizing of innate and adaptive responses that lead to protective versus pathologic anti-PRRS responses.
- Development of sample and data resources for use by the PRRS research community.

Introduction
Studies comparing the response of different pig breeds to PRRSV infection support the notion that genetic components influence pigs' responses to PRRSV infection. These studies formed the basis for the creation of the PHGC. In the words of animal genomics experts, this is a project that will produce samples to be used to develop a "deep phenotype" of anti-PRRS responses and ultimately, with the availability of transcriptome and proteomic data, an "ultra-deep phenotype" of PRRSV infection. Samples are retained for analysis in the future as improved technologies become available. Another unique aspect is the support of PHGC from the NPB, PRRS CAP, USDA NIFA and ARS, Genome Canada, universities, and private companies.

Objectives
Objective 1. Use genotyping and phenotyping tools to identify host genes that control resistance/ susceptibility to PRRSV infection. The first part of this objective is directed at collecting the samples and phenotypic data from a large number of experimentally infected pigs. Pigs are infected with a standard PRRSV isolate (NVSL 97-7985) and PRRS phenotypic response information (mortality, weight, virus load, and immune response) are collected for up to 42 days post-infection (dpi). Serum samples are tested for viral levels (PRRSV RT-PCR), circulating immune-related proteins (e.g. cytokines), and antibodies (total and neutralizing). Tonsils are collected at the time of sacrifice for future studies of viral persistence. Genotyping using pig SNP chips are

funded through a PRRS CAP grant (Lunney) with whole-genome association analyses (WGAS) led by Jack Dekkers at Iowa State University.

Objective 2. Characterize variation in response to PRRSV. Under this objective, multivariate analyses (principal components and partitional cluster analyses (J.P. Steibel, Michigan State University) are used to statistically assign pigs from the PHGC infection trials into at least four virus/weight categories, i.e., high virus burden/maximal growth (HvHg); high virus burden/reduced growth (HvLg); low virus burden/maximal growth (LvHg); and low virus burden/reduced growth (LvLg). Later studies will test for genetic markers and phenotypic markers associated with resistance (LvHg), tolerance (HvHg), and susceptibility (HvLg). These categories will facilitate the identification of genes and proteins that are involved in resistance and susceptibility to PRRSV infection and the characterize the timing of their expression.

Objective 3. Characterize the relative importance of different phenotypes and genotypes that predict the response to PRRSV infection. Traits that predict a particular response to PRRSV infection, such as high or low serum levels of a pre-infection cytokine or protein, levels post-infection, or the association of a response with a genetic marker, are needed to support applied breeding programs. Gene expression in blood RNA is analyzed for each group using microarrays, RNA-seq and qPCR. NPB grant #09-208 "Comparison of early immune responses of pigs which are genetically PRRS resistant/tolerant using a swine-specific immune protein (cytokine) multiplex assay" used the Luminex microsphere, or "multiplex," cytokine assay (developed through NPB grant #08-189) to quantify serum cytokine levels. Additional funds to support gene expression and protein marker studies are from Genome Alberta and Genome Canada funding. The results will be used to identify proteins, pathways, and genes that are capable of distinguishing pigs that quickly clear PRRS virus from the blood versus pigs that maintain a high viral load.

Supporting all objectives is a relational database that can be mined for phenotypic and genotypic information. This effort is led by Dr. Reecy http://www.animalgenome.org/lunney/index.php

Materials & Methods

A. Background: The plan for the PRRS Host Genetics Consortium (PHGC) was developed at three one-day NPB meetings (12/15/05; 2/23/06; 5/9/07) with input from PRRS CAP and NC229 disease researchers, NC1037/NRSP8 genome researchers, members of the NPB Swine Health and Animal Science Committees, veterinarians and the American Association of Swine Veterinarians (AASV), producers, and commercial partners representing breeders (PIC, Newsham), animal health, feed, and diagnostic companies. The rules for participation in the PHGC are determined based on a USDA cooperative research and development agreement (CRADA). However, the PHGC is sufficiently flexible to allow for additional collaborators and new plans for phenotypic and genotypic analyses. The first published report on results are described in Boddicker et al., 2012, Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci. 90:1733-1746. This report described a selectable marker that can be used to breed for pigs that show improved weight gain and reduced viral load.

B. Pig sources and infection model: The source populations are crossbred pigs from commercial lines with complete parentage and pedigree records; this enables detection of PRRS resistance associated combinations of genes and chromosomal regions based on the SNP genotypes. Each experimental run involves 200 pigs. In general, piglets (~6/litter) are from a limited number of sires mated with 2-3 dams/sire. Pigs for the project have been supplied by six genetic companies: Genus/PIC USA; Newsham Choice Genetics; Fast Genetics; Genetiporc, Inc.; Genesus Genetics and TOPIGS. With support from a USDA NIFA grant, two inbred lines (bred for high and low feed efficiency) from a herd managed by Jack Dekkers at Iowa State, were added. The inclusion of the Iowa State pigs created the opportunity to study the relationship between nutrition and immune response to infection. Weaned pigs for each trial are from farms free of PRRSV, *Mycoplasma hyopneumoniae*, and swine influenza virus (SIV). For each round of infection, a group of 200 piglets at 14-28 days of age are infected with a relatively virulent PRRSV strain, NVSL 97-7985. Later trials, PHGC10-14, included a

contemporary isolate, KS-06. Blood samples are collected at -6, 0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi. In addition to serum, whole blood is collected into Tempus tubes for virus and host transcriptome analyses. Pigs are weighed weekly and oral fluid samples are collected on a daily basis for the first 21 days and then weekly thereafter. A sample of tonsil is collected at the end of the study for analysis of PRRSV persistence (NPB grant #12-061). For PHGC 13 and 14, fecal samples were collected as part of a study to understand the effect of PRRS on feed digestibility (NPB grant 12-151, The effects of PRRSV infection in commercial pigs on growth performance, energy and nutrient digestibility). All serum, blood, oral fluid, and tonsil samples are processed, aliquoted, and sent to appropriate testing labs. Dead and moribund pigs are necropsied at the Kansas State Veterinary Diagnostic Laboratory (KSVDL). In support of the sample collection activities, new -80 freezers were purchased by both Kansas State University and BARC (without NPB funds) to aid in sample storage; similarly, six additional -20 freezers have been procured at BARC as well as a backup Siemens electronic security system for the set of 16 freezers (through ARS BARC funds).

In 2013, with funding from USDA, the laboratory model is being adapted to the field. This is a 5 year $3 million grant, headed by Jack Dekkers, on translational genomics.

C. Phenotypic analysis: The phenotypic parameters include measurements of weight, average daily weight gain (ADWG), viral RNA level in serum, total virus load over 42 days, total antibody, neutralizing antibody, and cytokine levels in serum. ADWG is calculated by subtracting the weight at the end of study minus the weight at the beginning, divided by 42 days. Viremia is measured using commercial PRRSV qRT-PCR assays (ABI and Tetracore commercial assays performed at the KSVDL and in the Rowland laboratory). The results are reported as the number of PRRSV templates per reaction. Virus load for each pig is calculated by measuring the area under the curve of PRRSV RNA concentration during the first 21 days. Serum levels of interleukin-8 (IL-8) and interferon-gamma (IFN-gamma) are measured using in-house Luminex bead assays or Fluorescent Microsphere Immunoassays (FMIA) (NPB grants #08-189 and #09-208). The cytokine measurements were expanded to early innate (IL-1b), IFN-alpha, T helper 1 (Th1) (IL-12), Th2 (IL-4), and regulatory (IL-10) immune responses and cell migration chemokine (CCL2) with new funds fromGenome Alberta and Genome Canada grants. As part of an NPB proposal #12-120, we added total and neutralizing antibody as new phenotypes. In 2012, we added feed digestibility as a new phenotype.

D. Genotypic analysis: A tissue, DNA, and RNA repository was established at USDA BARC (Lunney) for all tested pigs and from parents (if available). Detailed designs for SNP genotyping and GWAS have been established through a four year PRRS CAP-funded project, "PRRS CAP Host genetics: Characterization of host factors that contribute to PRRS disease resistance and susceptibility"; therefore, no funds were requested from the NPB for the SNP typing. The PRRS CAP funding supports the activities of numerous swine genome and PRRS experts as CoPIs (Lunney, BARC; Dekkers, Garrick, Rothschild, Reecy, Iowa State Univ.; Jiang, Washington State Univ.; Steibel, Michigan State Univ.; Pogranichniy, Purdue). DNA samples from every PHGC pig and available parent are genotyped with Illumina's Porcine SNP60 BeadChip. The USDA AFRI grant "Identifying porcine genes and gene networks involved in effective response to PRRS virus using functional genomics and systems biology," has additional swine functional genome experts as CoPIs with PI Lunney (Honavar, Tuggle, Iowa State Univ.; Jiang, Washington State Univ.; Ernst, Steibel, Michigan State Univ.; Pogranichniy, Purdue). This grant is using functional genomics and systems biology analyses to identify genes, networks and pathways that regulate anti-PRRSV responses and maintain growth in the face of PRRSV infection. Data from the first set of 70 Pigoligoarrays analyzing blood RNA gene expression from 0 to 35 dpi have been completed (Arceo et al., 2013 . Characterizing differential individual response to Porcine Reproductive and Respiratory Syndrome Virus infection through statistical and functional analysis of gene expression. Frontiers in Livestock Genomics. 3:321). During the past years, additional support for the PHGC was obtained from the project, Genome Canada 2010 Large Scale Applied Research Competition: Project 2209 "Application of genomics to improving swine health and welfare." Support includes the experimental infection and PRRS phenotyping and DNA genotyping of 1,000 additional pigs challenged with a different PRRSV isolate. Another grant, "Canadian Component of the PRRS Host Genetics Consortium" was received through the Applied Livestock Genomics Program (ALGP) supported by Genome Alberta and Alberta Livestock and

Meat Agency. This funding supported the remainder of the SNPchip analyses for PHGC trials 7 and 8 DNAs, and has used next generation sequencing to analyze RNA expression (RNA-seq) of selected PHGC RNA samples. It has helped to expand the Luminex protein work to cover more samples and more cytokines as well as transfer the Luminex technology to Canadian labs. PigGen Canada is supporting this effort.

D. PHGC database at Iowa State University: The PHGC database resides on the Iowa State Linux based computers with an added Microsoft server as a data interface for PHGC participants. A password protected website has been developed for data submission and retrieval (http://www.animalgenome.org/lunney/index.php) and is accessible to authorized individuals at different levels. All PHGC data collected through the NPB, USDA NIFA, and ARS funded projects will be available to project members (through secure linkage) and eventually to the general public in the form of refereed publications and other resources. The PHGC database is supported by the backbone of NRSP8 Bioinformatics funds and security. The PHGC relational database tracks data associated with each pig and each biologic sample for the thousands of pig samples collected over the course of the multi-year PHGC project. The database serves as a data repository for all pig genotypic data, including parentage information, date of birth, allelic information [the genotypic information, major histocompatibility complex (SLA) alleles and haplotypes, and SNP data], and all phenotypic information, e.g., results of all assays performed on each sample (e.g., viral, antibody and cytokine levels at each time point).

The rules regarding database access and data sharing are covered under the USDA CRADA.

Results

Objective 1. Use genotyping and phenotyping tools to determine if there are host genes that control resistance/ susceptibility to PRRSV infection.

Virus infection of pigs and collection of samples (Rowland, Kansas State University). In summary, the three years of support have resulted in: 1) the challenge of approximately 1600 pigs with a well-characterized PRRSV isolate, 2) collection of phenotypic information, and 3) performing the SNP and GWAS. As a match, pigs were supplied by PIC/Genus, Newsham Choice Genetics, Fast Genetics, and Genetiporc Inc. Infection of PHGC4, 5, and 6 were completed with the year 2 funding. Funding for Year 3 funding was specifically applied to infect 400 pigs (PHGC7 and 8).

Update of PHGC relational database. The website has once again undergone substantial changes. It now utilizes the Bootstrap package, which is a package of Cascading Style Sheets (CSS) and Javascript tools that allows for a cleaner and more efficient website environment. There are more improvements in the queue that would further utilize the Bootstrap package that would make the website even more user friendly. New features on the website include 1) an email page that will email all members of the project group that have an account with the website and 2) a status page that displays what data has been entered into the database for each trial. The backend of the website and the database have also undergone some significant changes. For the large datasets produced, e.g., SNP and RNA-seq data, a combined system of flat files and MySQL database table usage has been incorporated, which allows for compressed data storage (less storage space required for the same amount of data) and quicker data retrieval from the website. The web address continues to be http://www.animalgenome.org/lunney.

Information sharing and confidentiality. The USDA ARS Office of Technology Transfer (OTT) has developed a revised and updated CRADA MTA for all participants to cover the confidentiality of data sharing.

Measurement of PRRSV RNA. ABI donated 10,000 free PRRSV PCR assays which were used for PHGC 1 through 5. For Year 3, NPB provided support for to PRRSV RT-PCR on all serum samples from PHGC 6, 7, and 8 (approximately 6,000 samples). This has been accomplished.

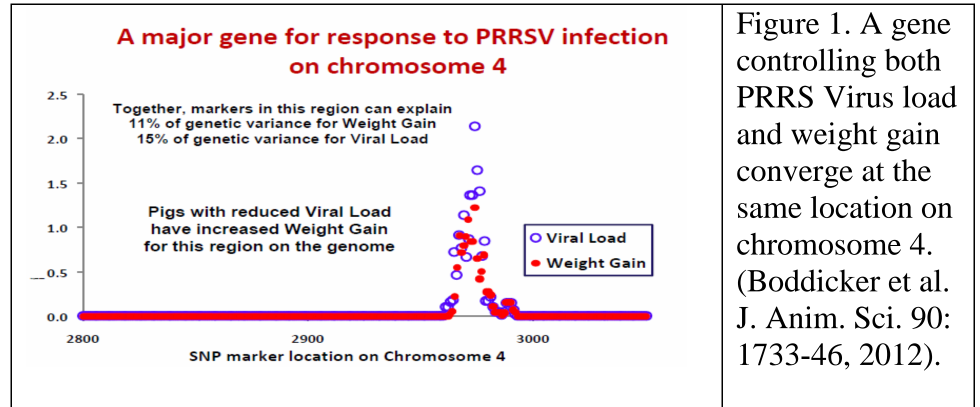

Figure 1. A gene controlling both PRRS Virus load and weight gain converge at the same location on chromosome 4. (Boddicker et al. J. Anim. Sci. 90: 1733-46, 2012).

Genome wide association studies (GWAS). All pigs in the study have been genotyped using the Illumina Porcine SNP60 BeadChip through matching funds. GWAS results (Nick Boddicker and Jack Dekkers, Iowa State) based on the weight and virus load phenotypes of the first 600 pigs. Genomic regions associated with virus load were found on chromosomes (SSC) 4 and X, and SSC1, 4, 7, and 17 for weight gain. A one Mb region identified on SSC4 influenced both weight gain and virus load as illustrated in Fig. 1. Genomic estimated breeding values (GEBV) for this region were very favorably correlated at -1. Candidate genes in this region on SSC4 include the interferon induced guanylate-binding protein (GBP) gene family, a group of genes involved in the inhibition of virus replication. In conclusion, host response to experimental PRRS virus challenge has a strong genetic component and a major QTL on SSC4 explains a substantial proportion of the genetic variance in the population of pigs studied so far. A model for how the favorable genotype influences virus load and weight gain is diagrammed in Fig. 2. The effect of the favorable genotypes AB or BB is a 10% increase in weight at 6 weeks post infection and corresponding decrease in virus load. The effect of the favorable allele is significant and will enable the application of marker-assisted breeding programs to reduce the impact of PRRS.

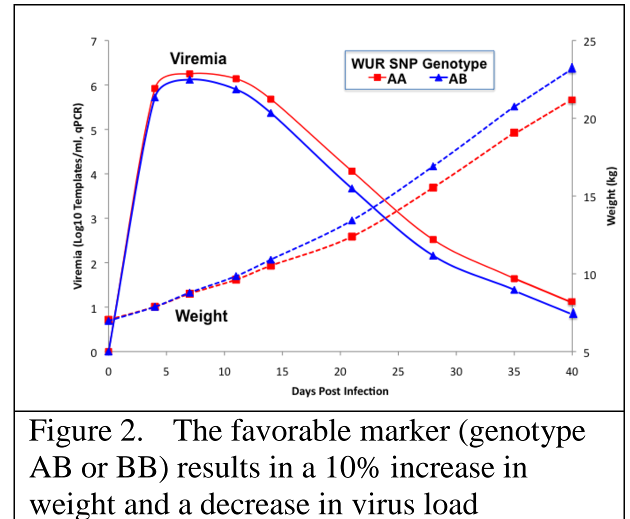

Figure 2. The favorable marker (genotype AB or BB) results in a 10% increase in weight and a decrease in virus load

A unique aspect of PHGC6 was the occurrence of a high rate of mortality that approached 50%. Based on necropsies the high mortality was linked to a broad number of bacterial infections, brought into the challenge facility by the pigs. Obviously, this group of pigs did not possess a “high health” status, but are typical of stressed pigs. Separate GWAS analyses at Iowa State (Boddicker, Dekkers) affirmed that two markers on chromosome 1, previously linked to virus load, were also associated with mortality. For one marker, all pigs that died possessed the marker; whereas, all pigs that survived lacked the marker. This marker could have significant predictive value in identifying pigs that succumb to the combination of PRRSV infection and other stressors. Such a marker would be useful to avoid such pigs if going to producers in pig dense (high disease stress) areas.

Even more recently is the characterization of a SNP marker linked to the PRRSV antibody response. This study, supported by NPB grant #12-120, examined serum virus N protein-specific total antibody levels measured by fluorescent microsphere immunoassay (FMIA). Serum and other data were collected from PHGC 1-3 samples. Heritability was 13.5±13.2%. The 1Mb region that explained the most genetic variation was found on chromosome 7 in a region harboring major histocompatibility (MHC) class I antigen genes, and was estimated to explain 54.2% of genetic variation. The results indicate the presence of a major QTL associated with anti-PRRSVIgG levels.

Objective 2. Characterize the genetic variation in response to PRRSV. During the first year, we identified a subpopulation of pigs that exhibited normal growth characteristics despite having an ongoing PRRSV infection. Such "PRRS tolerant" pigs would be particularly valuable for high density pig regions where PRRSV is endemic and difficult to control. Beginning in Year 2 and continuing through Year 3, multivariate analyses (principal components and partitional cluster analyses) were performed on PHGC weight and virus load data by Dr. JP Steibel, Michigan State University (additional support by USDA NIFA functional genomics grant). The results showed that subpopulations of pigs could be assigned into at least four virus/weight categories of PRRSV infected pigs, i.e., high virus burden/maximal growth or HvHg; high virus burden/reduced growth HvLg; low virus burden/maximal growth LvHg; and low virus burden/reduced growth or LvLg. Based on these assignments, later studies will test for genetic markers associated with PRRS resistance (LvHg), tolerance (HvHg), and susceptibility (HvLg), and prevent selection for PRRS resistance with poor growth (LvLg). These categories will facilitate genetic studies to determine genes and serum proteins (biomarkers) associated with PRRS resistance/susceptibility and associated growth traits as affirmed by the USDA NIFA grant (#2010-65205-20433) for gene expression work and NPB grant (#09-244) and the most recent Genome Alberta and Genome Canada grants for protein biomarker studies using the Fluorescent Microsphere Immunoassay (FMIA) or cytokine FMIA (supported by NPB grant #08-189). Data on serum cytokine levels on selected PHGC3, 5 and 7 samples is now being summarized by Dr. C Souza at BARC. Differential levels of serum interferon-alpha (IFNa), interleukin-8 (IL-8) and chemokine CCL2 early after PRRSV infection are correlated with low or high viral levels.

In Year 3, the cytokine FMIA was used to evaluate cytokine levels in oral fluid samples from pigs that were either experimentally vaccinated for, or infected with, PRRSV. The levels of IL-8 and IL-1β in oral fluids peaked at 15 dpi/days post vaccination. IL-6 and CCL2 were only expressed at detectable levels in infected pigs; whereas, IL-12 was expressed only in vaccinated pigs. The results support the use of oral fluid samples as a means to evaluate the immune responses of pigs vaccinated or infected with PRRSV. A manuscript describing this work (Araujo et al., 2013) is currently in revision.

In Year 3, microarray studies were continued to characterize genes and gene pathways involved in the different H/L subpopulation phenotypes. When RNAs were tested at multiple dpi, these studies identified genes related to Inflammatory response, Cell-mediated immune response and Lymphoid Tissue Structure and Development, as summarized by Arceo et al., 2013 (Characterizing differential individual response to Porcine Reproductive and Respiratory Syndrome Virus infection through statistical and functional analysis of gene expression. Frontiers in Livestock Genomics. 3:321). Based on those results further analyses targeted just 0, 4 and 7 dpi RNA samples so 40 samples from 3 trials could be analyzed. This more detailed data has been collected, with the USDA NIFA grant (#2010-65205-20433) funds, and statistical analyses are underway at Michigan State University.

Objective 3. Identify relative importance of different phenotypes, and their heritability, that predict response to PRRSV infection.

PHGC relational database. (see above for progress related to the database). With the new NPB grant #12-061 (Lunney, Reecy) and USDA NIFA translational genomics grant (Dekkers et al. 2013-68004-20362), the database will be expanded to include gene expression microarray and RNA-seq results on blood RNAs. All data collected through the project will be available securely to project members prior to publication and to general public after original publication.

Value-added spinoffs to the PHGC. As described in earlier progress reports, we took advantage of the infection of large number of pigs to support other aspects of PRRSV research. One value-added activity is the collection and analysis of oral fluids. These samples are be used to support a number of new projects, including new PRRSV diagnostic tests.

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Discussion

The PHGC has captured the imagination of the infectious disease and animal genetics communities and has emerged as the "first of its kind" and "one of a kind." The project has set the standard for the investigation of host responses to infection. Funding for the first three years has been primarily devoted to the infection of pigs, cataloging of samples, collection of phenotypic data, preparation of genomic DNA for genotyping, and the development of the PHGC database. The NPB Year 3 funding supported the infection groups, PHGC 7 and 8, support for the database and measurement of cytokine responses. A marker on chromosome 4 creates the opportunity to breed pigs for improved PRRS resistance. The project continues to accrue significant leveraging.