RESEARCH REPORT
SWINE HEALTH

Title: PRRS host genetics consortium (PHGC): a proposal to continue consortium work to study the role of host genetics and resistance to PRRSV – **NPB #12-061**

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Industry Summary:

Despite extensive efforts to eliminate PRRS from US production facilities, it remains a key disease issue and poses a continued economic threat to the industry, particularly in pig dense areas. A major factor that complicates PRRS control is viral persistence. This project proposed to identify pigs which have persistent PRRSV infections. Persistence is a major epidemiological feature of PRRS virus. Pigs with persistent PRRSV infections, or carrier pigs, are a continuing threat to each production unit. Viral survival is maintained because a proportion of the herd have persistent virus which is shed occasionally (due to other diseases or stress). This shed virus then infects the remaining herd pigs which are naïve and thus susceptible. Transmission studies have verified that pigs can harbor the virus for >160 days, and likely longer. Closing a herd for 200 days was thought to be effective before new "clean pigs" could be reintroduced; some would argue for longer times.

Currently there is no good technology to accurately identify PRRSV carrier pigs, nor are there procedures to treat pigs to eliminate persistent virus from their tissues. This proposal determined the frequency of pigs with persistent PRRSV by quantitating viral RNA levels in tonsil as a surrogate measure of viral persistence. To perform this we took advantage of the repository of samples that were collected through the NPB funded PRRS Host Genetics Consortium (PHGC). Each PHGC pig, provided at weaning from current commercial breeding stocks, was infected with a virulent PRRSV isolate (NVSL 97-7985) and followed for 42 days post infection (dpi). Every pig that survived to 42 dpi had tonsil tissue archived. The PHGC database (www.animalgenome.org/lunney/index.php) has extensive data on each PHGC pig, including its pedigree, response to PRRSV infection (serum viral levels and weight gain data), and extensive genotypic information (60K SNP chip). As a results of efforts for the 3rd objective of this grant the PHGC database has been updated and expanded. It now has the capacity to archive data from more trials (up to PHGC25) and handle new data types such as next generation, deep sequencing gene expression data, as well as to save draft manuscripts and slide presentations.

For Objective 1 of this grant, PHGC trials 3 and 5 RNA was carefully extracted from tonsil of every pig that survived to 42 dpi. That RNA was then tested for viral RNA using a sensitive molecular assay. The resulting data clearly show that there is high variability in tonsil viral levels at 42 dpi with PRRSV isolate (NVSL 97-7985) in nursery pigs. Using this surrogate persistence measure, especially with sets of similarly PRRSV

challenged pigs that have great variation in both serum and tonsil viral RNA levels, provided us with testable hypotheses to query for controls of persistence. Since sera from persistently infected pigs frequently are virus and antibody negative, these results and the PHGC data archive provided substantial means to affirm if there are any tissue or serological correlates of PRRSV persistence.

Our results affirmed that there is no correlation between tonsil viral RNA levels of pigs and 1) serum viral level at 42 dpi; 2) early serum viral levels (0-21 dpi); 3) weight gain changes (0-42dpi); or 4) immune gene expression in tonsil (limited survey). These results, while disappointing, were not unexpected given data accumulated from previous experiments.

These results set the stage for more detailed analyses. The wide variability of tonsil viral RNA levels opened up new avenues for querying factors that might be involved in tonsil virus persistence. Our new NPB grant (#14-223) will use sophisticated genome mapping techniques to determine whether there are genomic regions and host genes that regulate tonsil virus persistence. We will probe for mechanisms controlling tonsil PRRS viral levels using deep sequencing of tonsil RNA gene expression and statistical analyses. Comparing data from pigs with high versus low tonsil PRRS viral levels and using bioinformatic tools we hope to identify molecular pathways and genes involved in anti-persistence responses. With this knowledge, efforts can be planned to selectively breed for these pigs or, preferably, to identify means of stimulating these responses in pigs with high persistent PRRS viral RNA.

Keywords: PRRS, persistence, tonsil, viral RNA, database, host genetics

Scientific Abstract:

Despite extensive efforts to eliminate PRRS from US production facilities, it remains a key disease issue and poses a continued economic threat to the industry, particularly in pig dense areas. A major factor that complicates PRRS control is viral persistence. Viral survival is maintained because a proportion of the herd has persistent virus which is shed occasionally (due likely to other diseases or stress). This shed virus then infects the remaining herd pigs which are naïve and thus susceptible. This project proposed to identify pigs which have persistent PRRSV infections and to determine if there are immune or genetic correlates of PRRSV persistence.

Currently there is no good technology to accurately identify PRRSV carrier pigs. This proposal determined the frequency of pigs with persistent PRRSV using viral RNA levels in tonsil as a surrogate measure of persistence. To perform this we took advantage of the repository of samples that were collected through the NPB funded PRRS Host Genetics Consortium (PHGC). Each PHGC pig, provided at weaning from current commercial breeding stocks, was infected with PRRSV isolate (NVSL 97-7985) and followed for 42 days post infection (dpi). Every pig that survived to 42 dpi had tonsil tissue archived. Moreover the PHGC database (www.animalgenome.org/lunney/index.php) contains extensive data on each PHGC pig, including its pedigree, response to PRRSV infection (viral levels and weight gain data), and extensive genotypic information (60K SNP chip). As part of this grant the PHGC database has been updated and expanded to include capacity to archive gene expression data from microarrays and next generation sequencing data as well as manuscripts and slide presentations.

For PHGC trials 3 and 5 RNA was carefully extracted from tonsil of every pig that survived to 42 dpi. That RNA was then tested for viral RNA using a sensitive TaqMan assay. The resulting data clearly show that there is high variability in tonsil viral levels at 42 dpi with PRRSV isolate (NVSL 97-7985) in nursery pigs. Testing for tonsil viral RNA levels is not an ideal persistence measure since studies of long-term persistent virus have identified tissues with detectable viral RNA levels but which cannot transmit to naïve pigs. However, using this surrogate persistence measure, especially with sets of similarly PRRSV challenged pigs that have great variation in both serum and tonsil viral RNA levels, provides us with testable hypotheses to query controls of persistence. Since sera from persistently infected pigs frequently are virus and antibody negative, these results and the PHGC data archive provided substantial means to affirm if there are any tissue or serological correlates of PRRSV persistence.

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Introduction:

A major obstacle to the control and prevention of PRRSV infection in pigs is the capacity of the virus to become persistent within a production system (>167 days) (Wills et al., 1997; 2003; Rowland et al., 2003). As noted in a recent Extension update (Zimmerman et al., 2012), "Persistence is the single most significant epidemiological feature of PRRS virus. Carrier animals represent the constant threat of transmission to susceptible herd mates and the initiation of a PRRS outbreak. At present, we do not have the technology to accurately, rapidly, and cheaply identify carriers. Neither the absence of viremia nor serum antibody levels is an indicator of carrier status. Thus, the existence of carrier animals profoundly complicates all aspects of PRRS prevention and control." Moreover, because PRRSV is an RNA virus, its sequence randomly changes as it replicates, which results in viral genetic variants that can escape immunity in previously vaccinated pigs. Transmission studies have verified that pigs can harbor the virus for >160 days, and likely longer. Closing a herd for 200 days was thought to be effective before new "clean pigs" could be reintroduced; some would argue for longer times.

Over time, viral loads decrease by >1,000-fold in tonsil and lymph nodes, the primary sites of viral persistence (Xiao et al., 2004). Evans et al. (2010) developed a stochastic model of within-herd PRRSV transmission dynamics, which indicated a balance between "fade-out" and persistence. Molecular analyses revealed that viral RNA was present in tissues of infected pigs until 202 dpi in the ''Big Pig'' experimental samples (Molina et al., 2008). The mechanism of persistence is not completely understood, but likely includes the emergence of viral variants that can escape host defenses (Rowland et al., 1999). Horter et al. (2002; 2003) found that infectious PRRSV is present in most pigs for the first 105 dpi, and suggested that RT-PCR assays of oropharyngeal (tonsil) scrapings was the most effective combination of assay and sample for detecting PRRSV carriers. Thus tonsils were the focus of this proposal.

Horter et al. (2002) noted that antibody response levels did not distinguish PRRSV carrier from non-carrier animals. Mulupuri et al. (2008) suggested that the delayed response against the GP5 protein of PRRSV early in infection may contribute to the prolonged acute infection and the establishment of persistence. Analyses of the "Big Pig" samples indicated that upregulation of serum cytokines [interleukin-1β (IL-1β), IL-8 and interferon-γ (IFNγ)] early after infection was significantly correlated with lower lymph node or tonsil tissue viral RNA levels (Lunney et al., 2010). Rodríguez-Gómez et al. (2013) suggested persistence was due to impairment of the immune function of antigen presenting cells. This may explain the negative role PRRS has on co-infections, contributing to polymicrobial diseases such as Porcine Respiratory Disease Complex (PRDC) and porcine circovirus associated disease (PCVAD) (Opriessnig et al., 2011; Gómez-Laguna et al; 2013a,b).

Research has shown that there are genetic components involved in determining how pigs respond to PRRSV infection (Halbur et al 1998; Vincent et al 2005; 2006; Petry et al 2005; 2007; Ait-Ali et al 2007; Lewis et al 2007; Lunney and Chen, 2010). To elucidate clearly the role of host genetics in the control of PRRS requires a large numbers of animals. This recognition formed the basis for the creation of the PRRS Host Genetics Consortium (PHGC); which would 1) support and organize the infection of thousands of pigs, 2) collect, catalog and distribute tens of thousands of samples for analysis by PHGC participants, and 3) develop and maintain a consortium database (Lunney et al., 2011; Rowland et al., 2012). NPB provided critical early support of this effort (NPB grants #07-233, 09-208, 10-033).

Analyses of the first 8 PHGC trials of commercial pigs from 6 different genetic sources affirmed that serum viremia and weight gain after infection were moderately heritable at 0.39 and 0.34, respectively (Boddicker et al., 2014b). State-of-the-art genome wide association studies (GWAS) were performed and identified a region on swine chromosome 4 (SSC4) that is associated with PRRS resistance/susceptibility and explained 15.7% of the genetic variance for VL and 11.2% for WG42 (Boddicker et al., 2012; 2014a,b). These phenotypic observations help to explain the impact of PRRSV infection at the level of a population and why PRRSV is such a stealthy and pervasive virus. Now samples from these same PHGC pigs will be probed for PRRS persistence.

Objectives

This proposal has 3 objectives: 1) Determine levels of PRRS viral RNA in porcine tonsils of PHGC pigs to assess PRRS persistence; 2) Assess tonsil tissue RNA for immune correlates of PRRSV persistence; and 3) Expand capacity of PHGC database and improve public/private interface.

Materials & Methods:

As part of the original NPB funded PHGC grants (#07-233, 09-208, 10-033) tonsil samples were collected from every pig that survived until the end of the PRRSV infection trial, typically to 42 dpi. Pigs were brought into the Kansas State University (KSU) biosafety level 2 (BSL-2) facility at weaning (14-21 days age), infected a week later with a well characterized PRRSV isolate (NVSL 97-7985) and followed for 42 dpi. Whole blood and serum samples for RNA were collected on 0, 4, 7, 11, 14, 21, 28, 35 and 42 dpi and body weight of the animals were measured weekly. Tonsil, ears and other samples were collected at the end of the study. All samples were catalogued, shared between Kansas State University and BARC and stored for use in later studies or distributed to appropriate testing labs. Data on every pig was entered into the PHGC database (www.animalgenome.org/lunney/index.php). Genomic DNA samples from every PHGC pig and available parent was genotyped using the Illumina Porcine SNP60 BeadChip.

The PHGC tonsils were shipped from Kansas State University (KSU) to BARC and stored in -80 freezer until processed for RNA. Specifically, samples were kept on ice as a piece of tonsil (~30mg pea size) was removed. The tissues were homogenized using the FAST tissue prep tubes with lysis buffer and a steel ball at 30 Hz for 4 minutes total on Mixer Mill Model MM301 tissue grinder. The RNA was extracted with the Qiagen RNeasy protocol. The resulting RNA was aliquoted and stored in the -80 freezer. RNA quality and quantity was affirmed using an Agilent 2200 Tape station. The average total RNA yield was ~103 ug/ 90 uL (range 1.4 – 394 ug) with an average RNA quality score, known as RIN#, = 6.3 +/- 0.7 (range 4.4 - 8.2).

Viral copy numbers were quantified using One-Step quantitative qRT-PCR protocol on an ABI7500 Biosystems Real-Time PCR System. The viral qRT-PCR TaqMan assay used was the one developed in Dr. John Harding's lab at the University of Saskatchewan (Ladinig et al., 2015). It is specific for the NVSL 97-7985 PRRSV isolate and used their cloned standard qPCR Std (pCR2.1 TOPO-NVSL) for quantitation. Based on the threshold cycle (Ct) values from the standard curve, viral copies/ul RNA and /mg tonsil tissue were calculated. Samples with low, undetermined viral RNA levels (Ct >/= 39) were assigned as 1.0 copies/ul for graphs. All data generated in this project was stored in the PHGC database (www.animalgenome.org/lunney/index.php).

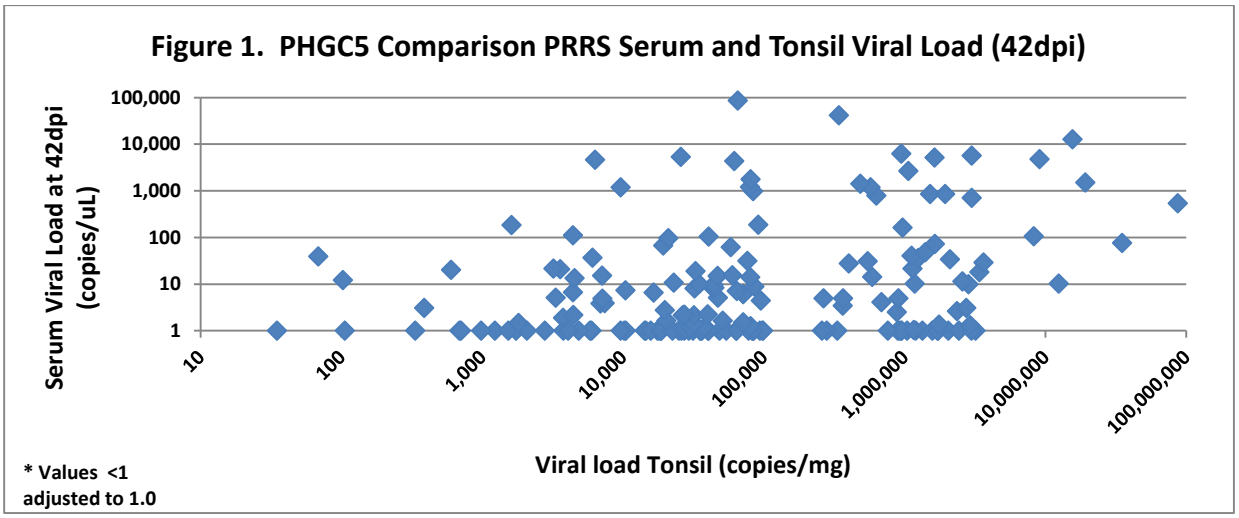
For Obj.2 tonsil RNAs were transcribed into cDNA using Superscript III (Life Technologies). cDNAs were assayed using immune qPCR techniques from the lab (Lunney et al., 2010). Expression levels of a limited set of immune genes were compared in RNAs prepared from tonsils of pigs with high versus low tonsil viral RNAs.

Results:

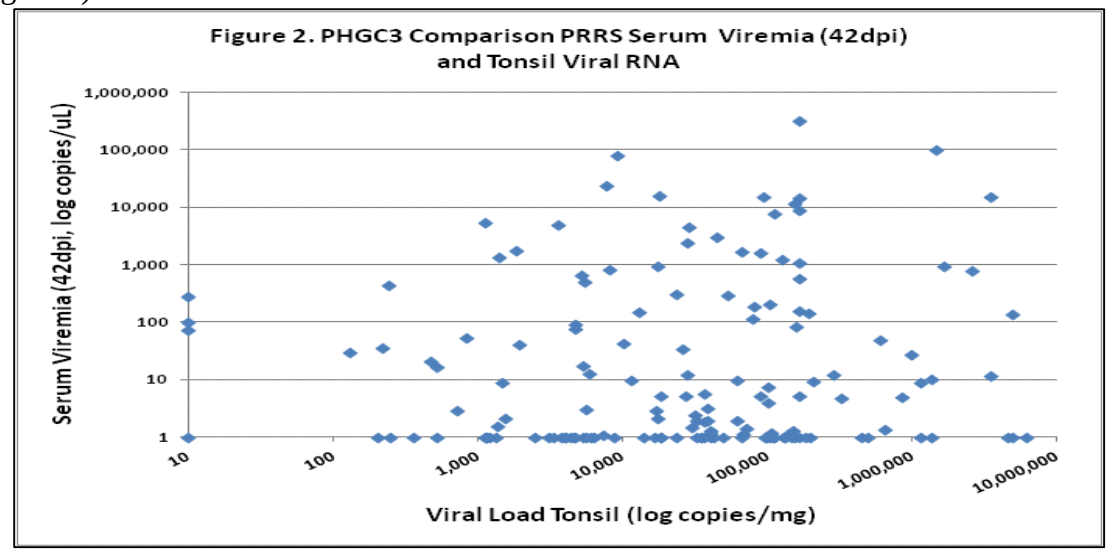
Obj. 1. Determine levels of PRRS viral RNA in porcine tonsils of PHGC pigs to assess PRRS persistence;

As proposed in our grant we screened tonsils from every pig in a few PHGC trials to affirm the % of pigs with persistent virus in the tonsil. To start we chose PHGC trial 5 (PHGC5) since it had a high number of pigs with detectable serum viral level, as measured by quantitation of viral RNA in serum at 42dpi. The sera of 15.3% of PHGC5 pigs (29/190) had viral quantity > 100 viral copies/ul at 42 dpi. The PRRSV TaqMan assay was used to assess the level of PRRSV RNA in each tonsil RNA prep. Results indicated that there was as low as no detectable viral RNA in some pig tonsils to a high of 32 million viral RNA copies/mg tissue. Figure 1

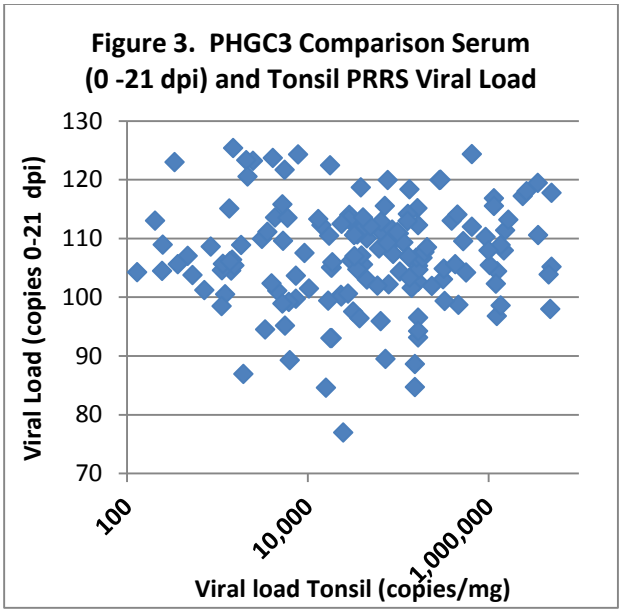
affirms that many pigs with low serum viral still had substantial levels of PRRSV RNA in their tonsils. There clearly is a lack of relationship between the 42 dpi serum viral level with tonsil viral RNA (copies/mg tissue). Pigs with no serum virus have widely varying levels of tonsil viral RNA (4-5 log difference in RNA copies).

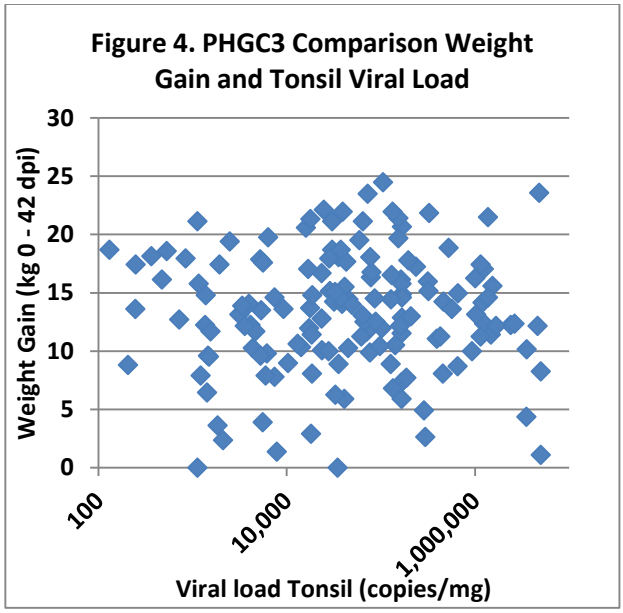


This lack of correlation between serum and tissue viral levels could be related to the detection of packaged virus RNA in serum, versus multiple copies of target RNA (genomic/ subgenomic not virus packaged) in tonsil cells that replicate the virus. Indeed, testing for tonsil viral RNA levels is not an ideal persistence measure since studies of long-term persistent virus have identified tissues with detectable viral RNA levels but which cannot transmit to naïve pigs. However, using this surrogate persistence measure, especially with sets of similarly PRRSV challenged pigs that have great variation in both serum and tonsil viral RNA levels, provides us with testable hypotheses to query controls of persistence. Since sera from persistently infected pigs frequently are virus and antibody negative this sample and PHGC data archive provided substantial means to affirm if there are any tissue or serological correlates of PRRSV persistence. Similar results were found for PHGC trail 3 (Figure 2).



We analyzed other factors for associations with tonsil viral RNA levels. First we asked whether early post infection serum viral load (VL from 0-21 dpi) would influence tonsil levels but found no associations (Figure 3). When weight gain was analyzed, there was a similar lack of correlation between weight gain from 0 to 42 dpi and tonsil viral RNA (Figure 4).





Overall, our data clearly show that there is high variability in tonsil viral levels at 42 dpi with PRRSV isolate (NVSL 97-7985) in nursery pigs. Unfortunately we could find no correlation between tonsil PRRS viral RNA levels in PHGC3 or PHGC5 pigs and 1) serum viral level at 42 dpi; 2) early serum viral levels (0-21 dpi); or 3) weight gain changes (0-42dpi). This was true for both all PHGC3 and PHGC5 data as well as for limited sampling from PHGC2 and PHGC7 tonsils.

This large variation in tonsil viral RNA levels opens the possibility that there are genetic, cellular or immune factors involved in tonsil virus regulation. Causes for this variation might be identified using immune and genetic tests. We have started to focus on immune, and gene expression associated, control factors in Obj.2 of this grant. Probing for host genomic control is a focus of our new NPB grant (#14-223). That grant will also extend our gene expression efforts as well as ask whether similar results are found with a heterologous PRRSV isolate.

Obj. 2. Assess tonsil tissue RNA for immune correlates of PRRSV persistence;

Tonsil RNAs include not only PRRS viral RNA, but also potential host genes that may be involved in establishing and maintaining the tissue viral infection. To assess host gene expression, tonsil RNAs were transcribed into cDNA. Our first target was to assess expression of a limited set of candidate immune genes: cytokines (interferon-α (IFNA), IFNG, interleukin-5 (IL-5), IL-8, IL-13, TNF), chemokine (CCL5) and regulatory markers (NFKB, IRF1, TGM3) using quantitative PCR of tonsil mRNA/cDNA.

Our analyses showed that there was active host gene expression in the tonsil tissue but the data indicated that no matter the level of PRRSV RNA in the tonsil there was a very similar level of expression for most of these immune genes tested. Our preliminary analyses indicated only limited differences when host gene expression levels were compared for tonsil RNAs from pigs with high (400,000 – 4,000,000 viral equivalents/mg) versus low (7,000 - 32,000/mg) PRRS viral RNA. However, more analyses need to be performed. Rather than continuing to assess just a limited number of genes, as done here, our new NPB grant will use RNAseq analyses to assess the full range of expressed genes in tonsil RNAs. These analyses will allow us to more fully probe for a wide range of genes that might be involved in controlling tonsil viral levels. Thus further cDNA qPCR studies were not performed.

Based on our limited results we could find no correlation between tonsil PRRS viral RNA levels and immune gene expression levels.

Obj. 3. Expand capacity of PHGC database and improve public/private interface

The PHGC relational database http://www.animalgenome.org/lunney/index.php has undergone significant updates as a result of this grant. It started with the internal private database with a redesign of the website in order to make data access more user-friendly and enhance search functions, as well as improve the overall look

of the website. In particular, the genotype search function has been improved to use a metadata and flat file system to dramatically increase speed of the searches. There has also been a single animal search section added to see what data is available on each animal. This concept is applied to the different trials in general as well.

The amount of data stored in the PHGC database has continued to increase as PHGC trials were completed and data produced and verified. Further, the database has been expanded to warehouse RNA-seq transcriptome expression data. What information is currently stored in the database for a trial is displayed on a single page termed "Data." To date, data from PHGC1 through 16 have been added to the database (Table 1). These data include pedigree, weight and viral load information, and genotype data from the SNP analyses as outlined in the table below.

Table 1. Data in the PHGC Database

PHGC Trial	Birth Date	Sex	Sire	Dam	Weight	Viral Info	60k SNP Geno-type	Steibel Classifi-cation	Neutral-izing Ab Info	Total Ab Info	Serum Cyto-kine
1	198	198	198	198	198	187	187	67	166	0	0
2	200	200	200	200	200	190	200	55	174	0	0
3	200	200	200	200	200	186	199	70	151	0	36
4	195	195	195	195	195	195	195	64	196	0	0
5	199	199	199	199	199	199	199	65	178	0	78
6	126	126	126	126	126	200	126	41	70	0	0
7	197	197	197	197	197	197	197	70	63	0	120
8	200	200	200	200	200	200	198	74	0	0	0
9	200	200	200	200	200	200	184	0	0	0	0
10	200	200	200	200	200	200	200	0	133	0	0
11	200	200	200	200	200	200	195	0	124	0	0
12	189	189	189	189	189	189	185	0	28	0	0
13	189	189	189	189	189	189	185	0	0	0	0
14	182	182	182	182	187	187	187	0	0	0	0
15	200	200	200	200	200	200	186	0	0	0	0
16	232	0	232	232	232	232	185	0	0	0	0

Additional functions added to the website include a page that is designed to handle Power Point slides of presentations made by consortium members. Many of these presentations will be available to the public. Other materials include secure sites for pre-publication sharing of abstracts and articles. On the public side there is now a news site as well as copies of all published manuscripts produced with PHGC data. Overall the PHGCdb remains a robust system for storing all data in a centralized location allowing access to users via the PHGCdb website.

After this grant the continued funding for the PHGC database, and the PHGC database manager's (Eric Fritz-Waters) salary, is being included in each new grant, such as the successful 2013-17 USDA-AFRI translational genomics grant. Importantly the USDA national animal genome program, NRSP-8, Bioinformatics coordinator project funds have been used to cover the cost of computational and hardware expenses associated with the PHGC project. New NRSP-8 project funds covered a server upgrade, on which the PHGC database is warehoused, as well as the purchase of additional hard-disk storage space to meet the needs of the PHGC.

The PHGCdb also underwent a change in terms of data access. A restricted access system was put into place. This system allows for restricted access to the data at the individual user level, meaning that each user has their own level of access to the data as determined by the group and legal agreements signed. There are now separate Cooperative Research and Development Agreements (CRADAs) for PHGC trials 1-10 and 11-15 as

well as a Material Transfer Research Agreement (MTRA) for PHGC trials 16-25. ARS manages these agreements for the PHGC.

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