

Identification of factors associated with virus level in tonsils of pigs experimentally infected with porcine reproductive and respiratory syndrome virus¹

Andrew S. Hess,[†] Joan K. Lunney,[‡] Samuel Abrams,[‡] Igseo Choi,[‡] Ben R. Tribble,^{||}
Melanie K. Hess,[†] Raymond R.R. Rowland,^{||} Graham S. Plastow,[§] Jack C.M. Dekkers^{†,2}

[†]Department of Animal Science, Iowa State University, Ames, IA 50011; [‡]USDA, ARS, BARC, Beltsville, MD 20705; ^{||}College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506; and [§]University of Alberta, Edmonton, AB, Canada T6G 2R3

ABSTRACT: Porcine reproductive and respiratory syndrome (PRRS) is one of the most important global swine diseases from both an economic and animal welfare standpoint. PRRS has plagued the US swine industry for over 25 yr, and containment of PRRS virus (PRRSV) has been unsuccessful to date. The primary phase of PRRS, tracked by serum viremia, typically clears between 21 and 42 d postinfection (dpi) but tonsils are a main site of PRRSV persistence and PRRSV can be detected in tonsils in excess of 150 dpi. Measuring tonsil virus (TV) levels at late stages of infection (6 to 7 wk postinfection) can be used to assess tonsil persistence, as levels of virus in tonsil at this time likely influence how long the virus will remain in the tissue. TV levels were measured on pigs experimentally infected with either the NVSL-97-7895 (NVSL; $n = 524$) or KS-2006-72109 (KS06; $n = 328$) PRRSV type 2 isolates across five trials. The objectives of this study were to (i) estimate the heritability of TV levels at 35 or 42 dpi; (ii) identify factors that affect TV level, including serum viremia; (iii) identify genomic regions associated with TV level; and (iv)

compare results for the two PRRSV isolates. TV level was lowly heritable for both isolates (NVSL: 0.05 ± 0.06 ; KS06: 0.11 ± 0.10). Level of TV was phenotypically associated with traits related to viral clearance from serum: pigs with low TV levels had an earlier and faster rate of maximal serum viral clearance, lower total serum viral load, and lower viremia level at 35 or 42 dpi. Although no genomic regions with major effects on TV level were identified, several showed some association ($>0.1\%$ of total genetic variance in the NVSL-infected dataset, the KS06-infected dataset, and the combined dataset). These regions contained the genes CCL1, CCL2, CCL8, HS3ST3B1, GALNT10, TCF7, C1QA/B/C, HPSE, G0S2, and CD34, which are involved in viral infiltration or replication, immune cell migration, and viral clearance from tissue. Results were similar between the two PRRSV isolates. In conclusion, selection for viral clearance traits in serum may reduce PRRSV persistence in the tonsil across PRRSV isolates. However, genetic correlations need to be estimated to determine whether this will be successful.

Key words: genetics, pigs, porcine reproductive and respiratory syndrome, tonsils

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating, persistent viral swine disease (Holtkamp et al., 2013). Painstaking efforts are necessary to return an infected herd to negative status. Pigs are

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²Corresponding author: jdekkers@iastate.edu

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expected to clear PRRS virus (PRRSV) from serum 3 to 8 wk postinfection (Wills et al., 2003; Hess et al., 2016), but some pigs can be PRRSV positive in tissues for 150 to 250 d post infection (dpi) (Allende et al., 2000; Wills et al., 2003). The tonsil is an active site of PRRSV replication (Rowland et al., 2003). Pigs with PRRSV present in tonsils after the acute phase of infection are classified as persistently infected (Benfield et al., 1998). They can experience a resurgence of circulating virus, triggering a secondary outbreak (Rowland and Morrison, 2012)—a major concern to naïve pigs introduced into the herd after the initial PRRSV outbreak. Identification and removal of persistently infected pigs could substantially help with containment of PRRS and improve herd health, but persistently infected pigs are often asymptomatic, hampering efforts to identify them despite high levels of PRRSV in tissues (Wills et al., 2003). Genetic selection of pigs that are more resistant to PRRS is one method to improve the health status of the swine herd (Lewis et al., 2007). Serum viremia was previously found to be under genetic control, with a major QTL, marked by the SNP WUR10000125 (WUR) (Boddicker et al., 2014b; Hess et al., 2016). We hypothesized that animals that clear serum viremia levels faster, and thus have lower serum viremia at 35 or 42 dpi, will also have a lower tonsil virus (TV) level at that time, and that TV has a heritable genetic component, similar to serum viremia level. The objectives of this study were to (i) estimate the heritability of TV; (ii) identify factors that affect TV, including serum viremia; (iii) conduct a genome-wide association study (GWAS) to identify genomic regions associated with TV, including the WUR region; and (iv) compare the results for the two type 2 PRRSV isolates, NVSL, and KS06.

MATERIALS AND METHODS

Study Design

Pigs used for this study were from five experimental PRRS trials (3, 5, 7, 11, and 14) of the PRRS host genetics consortium (PHGC) (Lunney et al. 2011), whose objective is to identify host genes or genomic regions associated with increased resistance or reduced susceptibility of pigs to PRRSV infection. The Kansas State University Institutional Animal Care and Use Committee approved all experimental protocols for the animal trials used herein. A detailed description of the design, data collection and molecular techniques used in the PHGC trials is in Lunney et al. (2011),

with additional information provided in Hess et al. (2016). Briefly, for each trial, ~200 crossbred weaner pigs were provided from a commercial breeding program in the United States and Canada. Trials 3 and 11, as well as trials 5 and 14 had the same genetic background. Within each trial, pigs were from a single high health farm and genetic background, except for trial 5, which included pigs from one genetic background but two farms. All source farms were free of PRRS, *Mycoplasma hyopneumoniae*, and swine influenza. Animals were transported to Kansas State University at weaning (average age 21 d) and randomly placed into pens of 10 to 15 pigs as they were unloaded. After a 7-d acclimation period, pigs were experimentally infected, both intramuscularly and intranasally (see Lunney et al. 2011), with 10^5 (TCID₅₀) of NVSL-97-7985 (trials 3, 5, and 7), a highly virulent PRRSV isolate (Truong et al., 2004), or KS-2006-72109 (trials 11 and 14), a more contemporary PRRSV isolate. NVSL and KS06 are 89% similar at the GP5 nucleotide sequence level (Ladinig et al., 2015). Blood samples were collected at -6, 0, 4, 7, 11, 14, 21, 28, 35, and 42 d postinfection (dpi). BW was measured at 0, 7, 14, 21, 28, 35, and 42 dpi. Pigs were euthanized at 42 dpi. Trial 7 ended at 35 dpi due to limits on facility availability. Tonsil samples were collected immediately after pigs were euthanized. Across the NVSL trials, 5% of pigs died or were euthanized for humane reasons before 35/42 dpi. Mortality rate was similar in the KS06 trials, with 7% pigs dying or euthanized before 42 dpi across the two trials. Dead pigs were necropsied and subsequent gross and microscopic pathology by a board-certified pathologist identified PRRS associated disease as the major source of mortality.

Serum Viremia and TV Levels

Serum viremia and TV levels were measured using a semi-quantitative TaqMan PCR assay for PRRSV RNA, as described in Boddicker et al. (2012), with samples run in duplicate using 96-well plates. Serum and tonsil assay results were reported as the \log_{10} of PRRSV RNA copies per milliliter of serum and per milligram of tissue, respectively. For the NVSL trials (3, 5, and 7), the Applied Biosystems AgPath ID NA and EU PRRSV reagents (AB assay) were used to measure TV, while the Tetracore US and EURO PRRSV Master Mix reagents (Tetracore assay) were used for the KS06 trials (11 and 14) because the AB primers failed to amplify cDNA of the KS06 isolate.

TV level data were available on 524 NVSL-infected animals and on 328 KS06-infected animals. The RNA integrity number (RIN) score of RNA extracted from the tonsil samples was determined by an Agilent bioanalyzer, which serves as a measurement of the quality of the RNA in a sample. Fourteen animals were removed due to having an unreadable RIN, which is interpreted as a low quality RNA sample (Schroeder et al., 2006). Virus levels were assigned to a sample using a standard curve when performing the TaqMan qPCR assay. This standard curve has a linear portion for which virus levels can be accurately measured, while samples falling in the nonlinear range are not accurately measured at the dilution used. Based on this and the 14 animals without RIN, in total 30 NVSL-infected animals and 26 KS06-infected animals were identified as having low-confidence measurements, including samples whose virus levels fell outside the standard curve (too high (28) or too low (19)) or for which no virus was detected (9) due to either assay failure or lack of virus in the sample. These animals were removed from the analysis.

To evaluate the consistency between the AB and Tetracore assays, tonsil samples from a subset of 35 NVSL-infected animals were also evaluated using the Tetracore kit. Results showed that TV from the Tetracore assay had a lower mean and smaller standard deviation between samples than TV from the AB assay but they were highly correlated at 0.87 (Appendix 2, Supplementary material). To convert TV from the Tetracore assay to the scale of the AB assay, Tetracore results were regressed on the AB results (Appendix 2, Supplementary material) ($R^2 = 0.76$), and the resulting linear regression equation was used to adjust the NVSL TV data for differences between the kits, in order to obtain the expected TV based on the Tetracore kit.

Genotypes

Ear tissue was collected from all pigs at death or euthanasia for DNA isolation. Tissue or DNA samples for trials 3, 5, and 7 were genotyped with the Illumina Porcine SNP60 Beadchip (Ramos et al., 2009) v1 (San Diego, California) at GeneSeek Inc. (Lincoln, Nebraska) and samples from trials 11 and 14 were genotyped with the Illumina Porcine SNP60 Beadchip v2 (San Diego, CA) at Delta Genomics (Edmonton, AB). Only SNPs that were included on both versions of the Illumina's Porcine SNP60 Beadchip were used for analysis. SNPs were also removed if they were uninformative

or would give unreliable results, i.e. fixed within trial, unmapped, or mapped to a sex chromosome in build 10.2 of the swine genome (<http://www.ncbi.nlm.nih.gov/genome/guide/pig/>, accessed August 13, 2015); this left 48,164 SNPs. For these SNPs, missing genotypes (0.19%) were assigned the average genotype (on a 0/1/2 scale) for animals in that trial for that SNP. This set of SNPs will be referred to as the 60k SNPs.

Modeling Viremia Profiles with the Wood's Function

Previous studies utilized the Wood's curve to model serum viremia profiles in the PHGC trials (Hess et al., 2016, 2018; Islam et al., 2013):

$$V(t) = a_1 t^{b_1} e^{-c_1 t}$$

where $V(t)$ is serum viremia on the \log_{10} scale at t . a_1 impact the overall viremia level across time, b_1 is an indicator of the initial rate of increase to peak viremia, c_1 is an indicator of the rate of decline after the peak and dominates the viremia profile at the later stages of infection. These three function parameters were estimated for each individual that had at least five time points using Bayesian inference with a likelihood framework, implemented using a Markov chain Monte-Carlo method (Islam et al., 2013). The raw viremia profiles of some pigs appeared bi-modal, so an extended Wood's curve was also fitted for each pig using the same methodology:

$$V(t) = a_1 t^{b_1} e^{-c_1 t} + \max\left(0, a_2 (t - t_0)^{b_2} e^{-c_2 (t - t_0)}\right)$$

where t_0 denotes the onset of the second phase of the profile, which is assumed to follow the same Wood's curve shape as the primary phase and is thus defined by a second set of Wood's model parameters. A pig was classified as experiencing viremia rebound based on the Akaike's information criterion (AIC) if the extended Wood's curve had an AIC that was more than 1.488 smaller than the single Wood's curve, which corresponds to the 95% significance level of the likelihood ratio test between these models (Islam et al., 2013). In total, 15.5% of NVSL-infected pigs and 4.6% of KS06-infected pigs were classified as rebound pigs. Previously, NVSL was determined to be more virulent, while KS06 was characterized as being more persistent in serum (Hess et al., 2016). A total of 45.2% of NVSL-infected pigs and 65.2% of KS06-infected pigs were classified as having persistent

PRRSV levels in serum, defined as having an estimated \log_{10} serum value that exceeded 1 at the end of the trial (Islam et al., 2013), as determined by the most appropriate fitted Wood's curve (single or extended) for that individual. The remaining animals were assumed to have cleared viremia from serum by the end of the trial.

Five curve characteristics were derived to describe individual viremia profiles using the estimates of the curve parameters ($\hat{a}_1, \hat{b}_1, \hat{c}_1$) of the single or the extended Wood's curve in order to elucidate the relationship between serum and TV levels, as described in Hess et al. (2016):

VL_{Total} = area under the Wood's curve from infection until euthanasia, i.e. 35 (trial 7) or 42 dpi (trials 3, 5, 11, and 14). VL_{Total} includes both the level of viremia and the extent to which viremia is maintained.

TP = time to (first – in the case of rebound)

$$\text{peak viremia : TP} = \frac{\hat{b}_1}{\hat{c}_1}$$

$$\text{PV = peak viremia : PV} = \hat{a}_1 \left(\frac{\hat{b}_1}{\hat{c}_1} \right)^{\hat{b}_1} e^{-\hat{b}_1}$$

$$T_{\text{max}} = \text{time to maximal decay : } T_{\text{max}} = \frac{\hat{b} + \sqrt{\hat{b}}}{\hat{c}}$$

V_{max} = virus level at maximum viremia : $V_{\text{max}} =$

$$\left| -\hat{a}\sqrt{\hat{b}} \left(\frac{\hat{b} + \sqrt{\hat{b}}}{\hat{c}} \right)^{\hat{b}-1} e^{-(\hat{b} + \sqrt{\hat{b}})} \right|$$

Other Response Phenotypes

BW was collected weekly on all pigs and used to interpolate daily weights as described in Hess et al. (2016). Fitted weights from 0 to 7, 7 to 14, 14 to 21, 21 to 28, 28 to 35, and 35 to 42 dpi (when available) were used for analysis. Total PRRSV N-protein IgG (measured as an S:P ratio) was also available on 207 NVSL (trials 3 and 5) and 156 KS06 (trial 11)-infected pigs that had TV level data, as detailed in Hess et al. (2018). Neutralizing antibody (NAb) titer data were available on 360 NVSL (trials 3, 5, and 7) and 157 KS06 (trial 11)-infected animals that had sufficient serum available at the end of the trial. To determine NAb, collected serum samples were assayed as described in Tribble et al. (2015) by

incubating a 200 50% tissue culture infectious dose ($TCID_{50}$) of the virus with 1:2 dilutions of serum and then transferring to tissue culture plates with confluent MARC-145 cells; PRRSV cytopathic effects were assayed 4 d later. The inverse of the highest serum dilution without cytopathic effects was recorded as the NAb titer, which ranged from <8 to >1,024. For statistical analysis, NAb response was converted to an adjusted \log_2 scale (0 to 8).

Statistical Analyses

Factors Affecting TV Levels. TV assays were run on 96-well plates, and plate was partially confounded with both pen and family—with individuals from the same pen tending to be run on the same plate and half or full sibs also often run on the same plate. To evaluate the effect of including plate in the model, the following model was fitted to the TV data with or without plate and separately for each PRRSV isolate, using ASReml 3.0 (Gilmour et al., 2009):

$$Y_{ijklmnopqrs} = \mu + \text{Tr}_i + P_j + S_k + A_l + W_m \\ + R_n + \text{RIN}_o + \text{An}_p + \text{Li}_q \\ + \text{Pen}(\text{Tr})_{ir} + \text{Pl}_s + \varepsilon_{ijklmnopqrs}$$

where Y is the dependent variable of TV level. Parity of the dam (P), classified as first, second, or later parity, sex of the pig (S), and trial (Tr) were fitted as a fixed class effects. To account for potential differences between rebound and nonrebound pigs based on serum viremia, the fixed class effect of rebound (R) was also included in the model. Age (A) and weight (W) of the piglet at infection, and the RIN of the tonsil sample were fitted as linear covariates. Random effects included animal genetic effects (An), litter (Li), Pen nested within trial ($\text{Pen}(\text{Tr})$), Plate (Pl), and ε as the residual. Due to the limited pedigree information and availability of genotypes on almost all animals, the 60k SNP genotype data were used to construct a genomic relationship matrix (G -matrix) among all animals, using the method of VanRaden (2008). The G -matrix also included relationships between animals from different trials and genetic backgrounds.

Forty-three animals that did not have plate information were not used in this analysis. Results showed that plate explained a large part of the variance when it was included (58% for NVSL and 15% for KS06) and that this variance was partitioned between animal, pen, and residual when plate was not included in the model (Appendix 1, Supplementary material). The effect of plate that is partitioned to the animal component when

plate is not included in the model is expected to be a combination of genetic and plate effects due to confounding between plate and family, but it is not known in what proportions. Thus, estimates of heritability are expected to be biased upward when excluding plate and downward when including plate. We decided to exclude plate from the model because including plate would remove genetic effects from TV and from genetic correlations of TV with other traits.

Estimates of the heritability of TV and of phenotypic associations of TV with other PRRS response traits were obtained from the model described above without plate effects. The relationships of TV with serum viremia characteristics, weekly weight gains, and antibody response were estimated by separately including each of these traits as a fixed covariate in the model. When estimating the association between TV and WUR genotype, WUR genotype was added as a fixed effect. The significance threshold for the aforementioned analyses was set to $P \leq 0.05$, and the coefficient of determination of the independent variable was used to assess the proportion of TV explained by the covariate.

Genome-Wide Association Study

The Bayes C method implemented in GenSel 4.73 (Fernando and Garrick, 2012) was used to test associations of 60k SNPs with TV based on the following model:

$$Y_{ijklmnopqt} = \mu + Tr_i + P_j + S_k + A_l + W_m + R_n + RIN_o + Li_p + Pen(Tr)_{iq} + \sum_{r=1}^s z_{ri} \alpha_r \delta_r + \epsilon_{ijklmnopqt}$$

where Y is the dependent variable of adjusted TV; P , S , A , W , R , RIN , Tr , and $Pen(Tr)$ are as defined above but all fitted as fixed effects because GenSel does not accommodate random effects other than SNP effects; s is the number of SNPs fitted; z_r is the vector of genotypes of SNP r for each animal (i) coded as -10 , 0 , 10 , α_r is the allele substitution effect for SNP r , and δ_r indicates whether SNP r was included ($\delta_r = 1$) or excluded ($\delta_r = 0$) in the model for a given iteration of the Monte-Carlo Markov Chain. The prior probability of $\delta_r = 0$ was set equal to $\pi = 0.995$. A total of 500,000 iterations were run, including a burn-in of 50,000. Breeding values were calculated for each nonoverlapping 1 Mb region (window) for each iteration to obtain a sample of the breeding value for each animal for each window. The total breeding value of an animal was computed as the sum of the animal's

window breeding values across the genome. The percent genetic variance explained by a window in a given iteration was calculated as the variance of the breeding values for that window across all animals divided by the variance of total breeding values, multiplied by 100. The estimate of the percent genetic variance explained by a window was the posterior mean percent genetic variance explained by that window across all iterations (excluding 50,000 burn-in) (Garrick and Fernando, 2013).

RESULTS

Factors Affecting TV Level

Genetic Parameters. TV level was estimated to be lowly heritable for both isolates, with both heritabilities not significantly different from zero (Table 1). Genetic correlation estimates could not be obtained due to lack of model convergence.

Isolate and Serum Viremia Status. NVSL-infected pigs had 43% higher TV than KS06-infected pigs ($P < 0.001$); however, after adjusting TV for NVSL to the Tetracore scale, NVSL-infected pigs had only 2% higher TV than KS06-infected pigs, and this difference was not significant ($P > 0.1$; Table 2). For subsequent analyses, the unadjusted TV was used for NVSL-infected animals but for comparisons between isolates, estimates can be rescaled to the Tetracore scale by multiplying the least square means (Table 2) by 0.6753 and adding 0.2157 and multiplying regression coefficients (Table 3) by 0.6753.

For NVSL, pigs that were classified as having persistent serum viremia levels had significantly higher TV than pigs that had cleared serum viremia ($P < 0.001$). Rebound animals had intermediate average TV, which was not significantly different from the average TV of cleared or persistent animals ($P > 0.1$). For KS06, the direction of the effect of serum viremia status on TV was the same as for NVSL but differences were not as significant (Table 2). Adjusting NVSL to their Tetracore equivalent resulted in differences between cleared and persistent animals that were similar to those for KS06-infected animals.

Table 1. Estimates of heritability for the log of tonsil virus level for two PRRSV isolates

Isolate	Heritability (s.e.)	Phenotypic SD
NVSL	0.05 (0.06)	1.03
KS06	0.11 (0.10)	0.89
Both	0.09 (0.06)	0.98

Litter variance was zero for all analyses.

Phenotypic SD includes the effects of trial, pen, animal, and residual.

Table 2. LS means (s.e.; number of animals) of tonsil virus levels by isolate, serum viremia status, and WUR genotype

Trait	Level	NVSL	KS06
Isolate	Unadjusted*	4.69 (0.09; 480)	3.27 (0.13; 302)
	Adjusted	3.38 (0.07; 480)	3.30 (0.10; 302)
Serum viremia status	Cleared	4.45 ^a (0.09; 203)	3.35 ^a (0.17; 94)
	Rebound	4.62 ^{a,b} (0.13; 71)	3.48 ^a (0.27; 13)
	Persistent	4.88 ^b (0.09; 206)	3.54 ^a (0.16; 195)
WUR genotype	AA	4.63 (0.09; 270)	3.47 (0.15; 237)
	AB	4.71 (0.10; 181)	3.43 (0.18; 59)
	BB	4.65 (0.21; 29)	3.19 (0.38; 6)

Unadjusted: original tonsil virus levels were analyzed as measured.

Adjusted: tonsil virus levels for NVSL were adjusted to their Tetracore equivalent.

Estimates within the same isolate and dataset that have different letters are significantly different from each other.

*Estimates for NVSL and KS06 were significantly different from each other ($P < 0.001$).

Serum Viremia. Associations of different serum viremia curve characteristics with TV were assessed based on regression coefficients and coefficients of determination (R^2 ; Table 3). Neither TP nor PV were significantly associated with TV for either the NVSL or KS06 data. An earlier and faster virus clearance (T_{max} and V_{max}) was, however, associated with lower TV for both NVSL- and KS06-infected pigs. After adjustment of NVSL to their Tetracore equivalents, the regression coefficients for T_{max} and V_{max} were more similar for NVSL- and KS06-infected pigs. Animals that had lower levels of serum viremia throughout the trial (VL_{Total}) or on the day that the tonsils were collected (v_{END}) had lower TV for both NVSL- and KS06-infected pigs (Table 3).

Weight Gain. Analysis of weekly weight gain did not reveal any associations with TV (Table 3). However, there was a marginally significant ($P = 0.04$) positive association of weight gain during the last week of the trial with TV, suggesting that pigs that gained more weight at the end of the trial had higher TV. However, this was only evident for NVSL-infected pigs.

Antibody Response. Both S:P and NAb response had significant associations with TV in KS06-infected pigs but not for NVSL-infected pigs (Table 3). The estimates of the regression

Table 3. Associations of tonsil virus levels with serum viremia characteristics, weight gain, and antibody levels for the NVSL and KS06 isolate data

Statistic	TP	PV	T_{max}	V_{max}	VL_{Total}	v_{END}	W7	W14	W21	W28	W35	W42	WGEND	S:P	NAb
NVSL	N	480	480	480	480	471	480	480	480	480	480	339	480	207	332
	Reg. coeff.	0.043	-0.183	0.094	-3.244	0.164	0.151	0.185	0.174	0.108	0.05	-0.022	0.117	-0.09	-0.006
	s.e.	0.038	0.14	0.021	0.64	0.036	0.087	0.123	0.137	0.121	0.102	0.128	0.055	0.31	0.038
	P -value	0.26	0.19	<0.001	<0.001	<0.001	0.09	0.14	0.21	0.38	0.62	0.86	0.04	0.78	0.86
KS06	R^2	0	0	0.05	0.05	0.04	0.01	0.01	0	0	0	0	0.01	0	0
	N	302	302	302	302	281	302	302	302	302	302	302	302	137	132
	Reg. coeff.	0.043	-0.145	0.048	-2.362	0.215	0.108	0.102	0.016	-0.035	-0.05	-0.054	-0.054	-0.93	0.103
	s.e.	0.035	0.125	0.02	1.004	0.055	0.122	0.156	0.154	0.132	0.115	0.104	0.104	0.40	0.044
	P -value	0.23	0.25	0.02	0.02	<0.001	0.37	0.51	0.91	0.79	0.66	0.6	0.6	0.02	0.02
	R^2	0.01	0	0.02	0.02	0.05	0	0	0	0	0	0	0	0.05	0.05

TP = time to peak serum viremia (days); PV = peak serum viremia (\log_{10} serum viremia); T_{max} = time to maximal decay rate of serum viremia (days); V_{max} = rate of maximal decay rate of serum viremia (\log_{10} serum viremia/d); VL_{Total} = total serum viral load (\log_{10} serum viremia*day); v_{END} = measured serum viremia at the end of the trial (\log_{10} serum viremia); W7 = weight gain from 0 to 7 dpi (kg); W14 = weight gain from 7 to 14 dpi (kg); W21 = weight gain from 14 to 21 dpi (kg); W28 = weight gain from 21 to 28 dpi (kg); W35 = weight gain from 28 to 35 dpi (kg); W42 = weight gain from 35 to 42 dpi (kg); WGEND = weight gain in the last week of the trial (kg); S:P = virus N-protein-specific IgG response at 42 dpi (log₂ endpoint titer).

coefficients suggest that animals that had a higher antibody levels at the end of the trial had lower TV, while animals with higher neutralizing antibody titers had higher TV.

Genome-Wide Association Studies. Genotype at the WUR locus did not have a significant association with TV for either isolate ($P > 0.6$; Table 2). The full GWAS identified no major QTL for either isolate separately or jointly (Figure 1). The 1 Mb window with the greatest association explained only 0.14% of the genetic variance. One Mb regions that explained $>0.1\%$ of genetic variance when analyzing each isolate separately and combined were further investigated for their biological relevance to PRRSV infection because they were identified in independent analyses (NVSL and KS06). In the conservative dataset, 21, 24, and 62 regions were identified that explained at least 0.1% of genetic variance in the NVSL, KS06, and combined data sets, respectively. Of these, eight regions were identified in all three analyses, of which seven had functionally relevant

genes within 1 Mb in either direction of the identified 1 Mb window (Table 4).

DISCUSSION

Effect of Assay on TV Level Data

In this study, TV of NVSL-infected pigs was based on the AB assay and TV for the KS06-infected pigs on the Tetracore assay. Ideally, the reported level of virus in a sample is invariant to the probe set that is used for measurement, i.e., a regression coefficient and an R^2 near 1 when comparing results from the two probe sets on the same animals. However, here, the regression coefficient and R^2 were 0.63 and 0.76, respectively, for results from the AB and Tetracore assays on the set of 35 animals. The resulting regression equation was used to put results from the AB assay on the scale of the Tetracore assay, and this was used to compare results from the NVSL- and KS06-infected pigs.

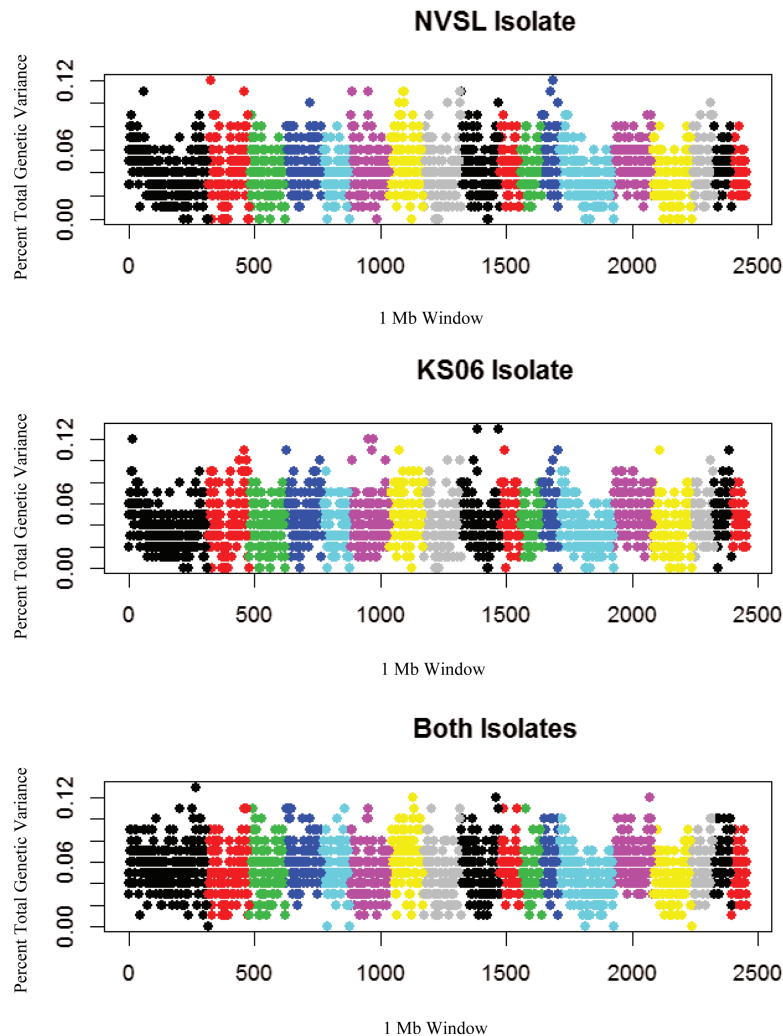


Figure 1. Manhattan plots for tonsil virus levels in pigs infected with NVSL or KS06 PRRSV isolates.

Table 4. Genomic regions and candidate genes associated with tonsil virus level identified by genome-wide association study (GWAS)

GWAS region (Chr_Mb)	Candidate genes	Relative location (Chr_Mb)	Function
12_42	CCL2		CCL2: displays chemotactic activity for <i>monocytes</i> and basophils
	CCL1	12_42.6	CCL1: <i>secreted by activated T cells</i> and displays chemotactic activity for <i>monocytes</i>
	CCL8		CCL8: chemotactic factor that attracts <i>monocytes</i> , lymphocytes, basophils, and eosinophils; this protein can bind heparin
12_60	HS3ST3B1	12_61.0	Catalyzes the addition of sulfate groups at the 3-OH position of glucosamine in <i>heparan sulfate</i>
16_75	GALNT10	16_74.6	Membrane-bound polypeptide that catalyzes the first step in mucin-type O-glycosylation of peptides in the <i>Golgi apparatus</i>
2_141	TCF7	2_142.0	Transcriptional activator— <i>T-cell lymphocyte differentiation</i>
6_74	C1QA/B/C	6_74.6	C1q associates with C1r and C1s in order to yield the first component of the <i>serum complement system</i>
7_39	?	7_39	
8_145	HPSE	8_144.3	Cleaves <i>heparan sulfate</i> proteoglycans to permit cell movement through remodeling of the extracellular matrix
9_147	G0S2	9_146.5	G0S2: promotes <i>apoptosis</i> by binding to BCL2
	CD34	9_148.4	CD34: important adhesion molecule and is required for <i>T cells</i> to enter lymph nodes; It also interacts with L-selectin, important in <i>inflammation</i>

All windows explained between 0.10% and 0.14% of genetic variance for GWAS including NVSL-infected animals, KS06-infected animals, or both

Genetic Parameter Estimates

Heritability estimates for TV were low and not significantly different from zero (Table 1). Bishop and Woolliams (2010) reviewed potential sources of variation in the macro environment that contribute to phenotypic variation, such as incomplete recording, incomplete exposure, and imperfect sensitivity and specificity of diagnosis, and showed that, if not controlled for, these factors have the potential to underestimate heritability. This may also be occurring with regard to the microenvironment when measuring virus levels in tonsils. Tonsils are a site of PRRSV replication and PRRSV preferentially replicates in macrophages. Thus, potential sources of variation that impact TV levels include the size of tonsils and the site at which the biopsy was taken (partial tonsil biopsies were taken in this study rather than whole tonsils), as well as macrophage abundance in the tonsils. If available, accounting for these factors as covariates in the model is expected to increase heritability estimates.

Our measures of TV capture both genomic and subgenomic RNA because the probes used in the TaqMan assays measure the amount of particular segments of RNA, rather than the whole strand of genomic RNA. Estimates of genetic correlations between traits give an indication of the genetic progress that can be made in a more difficult to measure trait (e.g. TV levels) by selecting on an easier trait to measure (e.g., serum viremia). Unfortunately, genetic correlations could not be calculated for

these data due to convergence issues, likely because of the low heritability of TV. In studies involving the same animals used in this study, heritability estimates of curve characteristics of serum viremia curve were moderate–high (Hess et al., 2016), so if serum viremia clearance characteristics are genetically correlated with TV levels, selection on those serum viremia characteristics may decrease viral persistence in tonsils.

Association of TV Levels with Host Response Traits

Estimates of regression coefficients of serum viremia traits on TV provided evidence that there is a relationship between serum viremia and TV levels, although these relationships were weak (low R^2) (Table 3). The results, however, suggest that faster clearance of virus from serum was associated with a lower level of virus that localizes to the tonsil, most likely because a lower level of circulating virus in serum affords the virus less of an opportunity to localize to tissues in which it persists. To our knowledge, this is the first time that such a relationship has been assessed for PRRSV infection. The relationship of TV with clearance of PRRSV from serum is of particular interest because these serum viremia characteristics explained the most variation in TV levels of all PRRSV response traits investigated when considering both isolates (Table 3).

Weight gain (WG) was not significantly associated with TV levels, with the exception of final WG in NVSL-infected pigs (Table 3). This is consistent

with the observation that persistently infected pigs are generally asymptomatic (Benfield et al., 1998; Wills et al., 2003).

A dynamic relationship has been observed between antibody response measured by S:P at 42 dpi and serum viremia levels throughout the course of infection (Hess et al., 2018). The phenotypic relationship between S:P and TV, both at 42 dpi, was negative following infection with either NVSL or KS06 but the association was only significant for KS06-infected animals. The stage of infection at 42 dpi may be different between NVSL- and KS06-infected pigs, because a greater proportion of NVSL-infected pigs cleared serum viremia before 42 dpi compared to KS06-infected pigs. To gain a deeper understanding of the relationship between TV and antibody levels, the relationship between these two traits needs to be investigated at multiple time points during infection.

A positive association was identified between NAb and TV for KS06-infected pigs but not for NVSL-infected pigs. This positive association may be due to antibody-dependent enhancement, a phenomenon in which virus-specific antibodies enhance entry and in some cases replication of virus in monocytes/macrophages and granulocytic cells through interaction with Fc and/or complement receptors (Yang et al., 2000). Antibody-dependent enhancement has been demonstrated for PRRS and has been shown to differ between virus isolates (Yoon et al., 1997). Neutralizing antibody titer was assessed using the inoculum but it is possible that the PRRS virions that are represented in the tonsil are immune escape variants (Evans et al., 2017) and that the antibodies that were previously neutralizing aid the population of PRRSV in the tonsil. Therefore, in addition to differences in results between NVSL and KS06 due to the timing of TV collection in relation to clearance of virus from the serum, these two virus strains may differ in their ability to enhance viral replication in the tonsils via antibody dependent enhancement. The negative (favorable) relationship observed for S:P with TV and the positive (unfavorable) relationship for NAb and TV could represent different levels of host-pathogen interaction; non-neutralizing antibodies could reduce viremia through C1q complement mediated responses (Lu et al., 2018) as discussed further below, whereas NAb could exacerbate virus levels due to antibody-dependent enhancement (Tirado and Yoon, 2003).

Genome-Wide Association Studies

In previous studies using animals included in this study, a major QTL was identified by GWAS,

which is tracked by the SNP WUR10000125 (WUR), for which animals that carry at least one copy of the B allele had reduced serum viremia and increased weight gain during PRRSV infection (Boddicker et al., 2012; Hess et al., 2016). A mutation in the GBP5 gene was identified as the putative causal for this QTL by Koltes et al. (2015), which is a gene that is involved with formation of the inflammasome, mediated by NLRP3 (Shenoy et al., 2012). This QTL was estimated to explain 13.4% and 7.5% of the genetic variation for serum viral load in NVSL- and KS06-infected pigs, respectively (Waide et al., 2018). However, genotype at the WUR locus did not have a significant association with TV for either isolate ($P > 0.6$; Table 2), suggesting a different biological mechanism for the prevalence of PRRSV in the tonsils.

The GWAS did not identify QTL with large effects. Nevertheless, eight genomic regions explained at least 0.1% of the genetic variance in the GWAS for each isolate and in the combined dataset. Genes that encode proteins that are responsible for modification of heparan sulfate, HPSE and HS3ST3B1, were located in the identified regions (Table 2). Heparan sulfate is a proteoglycan with covalently attached glycosaminoglycans (GAGs) and was the first identified mediator of PRRSV infection (Delputte et al., 2002). Virions are thought to concentrate on the surface of macrophages by attaching to GAGs, which stabilizes them and permits more efficient infection of the cell (Delputte et al., 2005). Heparanase is a hydrolase that cleaves between the saccharide subunits of the glycosaminoglycan chain, is expressed in immune-related tissues, and is upregulated in pigs infected with PRRSV (Tao et al., 2013). In vitro treatment of cells with HPSE has been shown to reduce PRRSV levels in infected porcine alveolar macrophages (Delputte et al., 2002). Heparanase activity is present in both endosomal and lysosomal compartments but can also occur at the cell surface, where it might digest adjacent heparin sulfate proteoglycans (Iozzo, 2001). The HS3ST3B1 gene is involved in GAG sulfation of the 3-OH position of glucosamine heparan sulfate, one of the final steps in biosynthesis of HS (Liu et al., 1999). Thus, the ability of the animal to modify heparan sulfate may be an area of interest for future studies that focus on susceptibility of the host to succumb to PRRSV persistence in the tonsil.

GALNT10 is another gene that is located in a region that was identified in the GWAS (Table 2). The short cytoplasmic tails of all GalNAc-Ts contain basic residues that may interact with peripheral Golgi membrane protein tethering complexes

(Smith and Lupashin, 2008). Upon entry into the macrophage, PRRSV particles migrate to the vesicles of the endoplasmic reticulum and the Golgi apparatus (Thanawongnuwech et al., 1997), where they then rearrange host cell membranes to establish a viral replication complex. The latter plays a structural and/or functional role by offering a suitable microenvironment for viral RNA synthesis, or facilitates recruitment of membrane associated host cell proteins for the purpose of viral replication and transcription (Pedersen et al., 1999). Thus, GALNT10 may be involved in establishment of the replication complex.

Other genes that are located in regions that were identified in the GWAS (Table 2) play a role in recruitment and activation of cells involved in immune response related to T-cell responses, including transcriptional activation of T-cell differentiation (TCF7), recruitment of monocytes (which differentiate into macrophages) by chemotactic cytokines such as CCL1 (secreted by activated T-cells), CCL2, and CCL8, and T-cell migration into lymph nodes (CD34). The genes C1QA/B/C are also located in one of the eight regions identified in the GWAS. C1q is the first subcomponent of the classical pathway of activation of complement. Both immunoglobulin (IgG and IgM) and viruses are known to interact with C1q (Kishore and Reid, 2000), which has been identified as a mediator of the clearance of apoptotic cells by macrophages (Taylor et al., 2000). Therefore, the ability of an animal to recruit T-cells to the site of infection and to signal uninfected macrophages to the site of infection may play a crucial role in containing PRRSV infection in the tonsils by targeting infected cells for clearance by macrophages via C1q.

CONCLUSIONS

This study evaluated factors that could be used to reduce PRRSV persistence in infected swine herds. TV levels at 35 or 42 dpi were estimated to be lowly heritable following infection with either NVSL or KS06 and no major QTL were identified for either PRRSV isolate. However, several genomic regions that explained a proportion of genetic variance and that harbored strong candidate genes were identified. Identified genomic regions included genes involved with the host's ability to control viral infiltration/replication, as well as the ability to clear infected cells from tissue. These genes may be useful targets for future gene expression analyses on tonsil tissue in order to gain insight into the host genetic control of TV levels and viral persistence. Clearance of viremia from serum and serum viremia levels at 35 or 42 dpi was phenotypically associated with TV

levels. The results from the GWAS analyses suggest that TV levels are likely regulated by both the host's ability to control viral infiltration/replication, as well as its ability to clear infected cells from tissue, which may be associated with serum viremia levels. This suggests that there may be a genetic correlation of TV level with T_{\max} , V_{\max} , or v_{END} , in which case selection that reduces serum viremia late in infection may also decrease TV levels and persistence. If serum viremia clearance characteristics are genetically correlated with TV levels, selection on those serum viremia characteristics could decrease viral persistence in tonsils.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. None declared.

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