RESEARCH REPORT
SWINE HEALTH

Title: Genomic Quasispecies Associated with the Persistence of PRRS Virus – **NPB #01-102**

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II. Abstract: An important issue for the control of PRRS is the persistence of PRRS virus for extended periods of time following infection of pigs and the lack of methods to detect persistently infected pigs. Previous studies indicate that lymphoid tissues and tonsil are preferred tissue sites for the virus to persist. In this study, PRRS virus was found to associate with lymph nodes within 6 to 24 hours post-inoculation and precede detection of virus in other tissues such as lung. This indicates that lymphoid tissue is the primary site of PRRS viral replication and that the virus establishes residence in lymph nodes early in infection. During acute infection, which is defined as the period to 28 dpi, virus isolation or RT-PCR is both adequate methods for detection of virus. Lung is the traditional tissue used for diagnosis of acute PRRS virus infections and virus was isolated as frequently from this tissue as most lymph nodes during the acute phase. Virus was more frequently isolated from palatine tonsil ($p<.05$) than lung and lymph nodes during the acute phase. The number of isolations of infectious virus from lymphoid and non-lymphoid tissues dropped markedly after 28 dpi. From 43 to 126 dpi RT-PCR is the diagnostic test of choice as infectious virus is rarely isolated from lymphoid or non-lymphoid tissues. Viral RNA was detected most frequently from 43 to 126 dpi in palatine and lingual tonsils compared to lymph nodes and other tissues. The early association of PRRS virus with lymph nodes may also explain why the virus predisposes pigs to secondary infections and provide a means for the virus to escape elimination by the immune system. The role of viral variation (quasispecies) as a mechanism for establishing persistence indicated that the most frequent change was a point mutation on nucleotide 97 on the ectodomain of the ORF5 gene that results in an amino acid change from glycine to serine at amino acid 33. The significance of this mutation was not determined in this study, but it does exist in a potential glycosylation site on the ORF5 gene. Tissue tropism does not appear to be related to a particular viral quasispecies.

III. Introduction: Porcine reproductive and respiratory syndrome (PRRS) is the most economically important swine disease in the world and is now endemic in most swine-producing countries. The clinical definition of the syndrome has expanded to include pneumonia in nursery and grow/finish pigs. In addition, PRRS infections of neonatal, nursery and grow/finish swine predispose these animals to co-infections with *Streptococcus suis, Salmonella cholerasuis* and *Mycoplasma hyopneumoniae*. Mycoplasma *hyopneumoniae* may also potentate PRRS pneumonia. Thus, economic losses due to PRRS are the result of either single agent infections by the PRRS virus or coinfections with various bacteria and other viruses.

PRRS is caused by a single-stranded, positive sense RNA virus initially isolated by investigators in the Netherlands (Lelystad virus) and later by our laboratory in collaboration with private industry (VR-2332). This virus is a member of the family *Arteriviridae* in the order *Nidovirales*. Biologically, the arteriviruses, replicate in macrophages and establish acute and persistent infections in their natural host. Persistence of PRRSV in herds and the inability to detect persistently infected pigs is now considered to be the major impediment to adequate control of this disease.

Field studies suggest that PRRSV can persist in pig nurseries up to 2.5 years after the initial outbreak. Sows are reported to shed PRRSV up to 99 days post inoculation (dpi) as determined by seroconversion of sentinel contact pigs. In another study, serologically naïve pigs exposed to pigs infected with PRRS virus 22 weeks previously, developed clinical PRRS, became viremic and developed antibodies to the PRRS virus. The virus has been detected in the oropharyngeal scrapings of 3/4 pigs on days 56, 70 and 84 dpi and in 1/4 pigs at 157 dpi. Recently, these studies have been extended and 20/28 pigs were found to be positive for PRRSV RNA by polymerase chain reaction (PCR) in tonsil at 84 dpi and only 1/28 at 119 dpi. Viral RNA was also detected in serum and tonsil by PCR through 251 and 225 dpi. Our research group has found that sera of pigs transplacentally exposed to PRRS virus at 85 to 90 days of gestation contain PRRS viral RNA at 210 days after farrowing. However, virus was more frequently recovered from either tonsil or lymph nodes than serum or other tissue. In addition, when we commingled naïve pigs with carrier pigs, we could demonstrate transmission of virus to sentinels up to 112 days after farrowing. Although pigs support the persistence of the PRRS virus for extended periods of time, the mechanism of persistence, the site of persistent virus replication and a method to detect persistently infected pigs is still not determined. However, information from studies on persistence indicates that tonsil and lymph nodes are frequent sites of infectious PRRS virus and viral RNA in persistently infected pigs. These tissues also appear to be frequent sites of replication during acute infection.

We have recently described the emergence of a PRRS viral variant with an amino acid change on the ORF5 hypervariable region of the genome that resulted in a new glycosylation site. These studies suggest that lymphoid tissue such as tonsil may be an ideal compartment to support variants of the PRRSV virus that are able to persist in the host and the potential to evade neutralizing antibodies. RNA viruses, such as PRRS virus, exist as a population of closely related viruses termed quasispecies. Quasispecies may play a role in tissue tropism and persistence. Our principal hypothesis is that the emergence of novel viral quasispecies in the lymphoid tissue of persistently infected animals allows PRRS virus to persist for varying time periods following infection.

IV. Objectives: The original objective was modified to: 1) Determine target tissue sites of acute and persistence PRRS virus replication in pigs infected at three-weeks of age, 2) Evaluate the utilization of conventional methods for the detection of infectious PRRS virus (VI), viral antigen (IHC), viral nucleic acid (nRT-PCR) and antibodies (IDEXX-ELISA and VN) in nursery age pigs from 6 hours to 126 days post inoculation, and 3) Evaluate if quasispecies evolution was a mechanism associated with viral tropism and persistence in pigs.

V. Materials and Methods: *Animals.* Seventy-seven 3-week old high health pigs were received from a commercial herd free of PRRS virus and use of PRRS vaccines. Upon arrival (0 day post-inoculation; dpi) the pigs were clinically observed and randomly divided into 4 experimental groups. The mock inoculated group

(n=15) of pigs were intranasally inoculated with 1ml/naris of minimal essential media. The remaining 62 pigs were randomly assigned to 3 separate isolation rooms in groups of 21, 21 and 20 pigs with each pig being intranasally inoculated with 1ml/naris (105.5 TCID50/pig) of the 92-23983SD strain of PRRS virus. Pigs were fed a standard commercial ration and had access to water *ad libitum*. All experimental procedures were approved by the SDSU Institutional Animal Care and Use Committee.

Virus and cells. MARC-145 cells (Kim et al. 1993. Arch Virol 133:477) were used to propagate the 92-23983SD strain of PRRS virus, for virus isolation and were used as indicator cells in virus neutralization tests. Cells were maintained as previously described (Benfield et al. 1992. JVDI 4:127; Rowland et al. 1999. Virology 259:262). In this experiment, the fourth cell culture passage of the 92-23983SD PRRS virus with a titer of 1.5x105 TCID50/ml was used.

Nested RT-PCR. Total RNA extraction was done using 300ul of serum or homogenized tissue, 900ul TRIzol® and 200ul chloroform according to manufacturer's (invitrogen™) recommendations. The aqueous phase was removed, the RNA was precipitated with 1.5 volumes isopropanol, RNA pellet was washed with 70% ethanol, pellet was allowed to dry at ambient room temperature and then reconstituted in 20 ul of RNase and DNase free distilled water. Nested RT-PCR amplification of ORF 7 was done as previously described by Christopher-Hennings et al. (1995. J Clin Micro 33:1730).

Virus Isolation. A 10% w/v suspension of tissue or 100µl of serum were used as starting material to prepare 10-fold serial dilutions (10-1 to 10 -6). Each dilution (200µl) was added to triplicate wells on 96 well plates of MARC-145 cells and incubated for 4 days. Plates were then fixed in 80% acetone and stained with FITC-SDOW-17.

Histopathology and Immunohistochemistry. Tissues collected at necropsy were fixed in 10% neutral buffered formalin, embedded, sectioned and stained with hematoxylin and eosin. Immunohistochemical staining of paraffin-embedded 6-µm sections was done using the anti-nucleocapsid monoclonal antibody SDOW-17 (Nelson et al. 1993. J Clin Micro 31:3184) and the commercial DAKO EnVision+System and associated reagents.

Measurement of Antibody and Neutralizing Activity. PRRSV-specific antibody in serum was measured using the HerdCheck® PRRS ELISA (IDEXX) according to the manufacturer's instructions. An S/P ratio >0.39 was considered seropositive. PRRS virus neutralizing activity in serum was measured according to Rowland et al. (1999. Virology 259:262). Serial 1:2 dilutions of serum were prepared in culture medium on 96 well plates. An equal volume of the 92-23983SD PRRS virus at a concentration of 20,000 TCID50/ml, was added to each sample, incubated for one h at 37^{o}C, and then transferred to a 96 well plate containing confluent MARC-145 cells. After 24 hours the plates were fixed in 80% acetone and infected cells detected with FITC-labeled anti-nucleocapsid monoclonal antibody SDOW-17. Neutralizing activity was reported as the log of the highest serum dilution that prevented the replication of PRRS virus in MARC-145 cells (negative for SDOW-17 staining).

PRRSV Quasispecies. The PCR amplicons were purified using the Wizard Prep kit (Promega) and cloned in the pCR-Blunt II-TOPO vector (Invitrogen). Multiple clones were sequenced using vector-specific primers. Cycle sequencing was performed with fluorescent
Big Dye chain terminators (Applied Biosystems) and sequencing products were submitted to the Iowa State Sequencing Facility for sequence determination using an ABI 377 automated DNA fragment analyzer. Sequence data were assembled and analyzed using the GeneTool sequence analysis program (BioTools, Inc., Edminton, CA). Multiple sequence alignments were
generated using CLUSTALX.

Experimental Design. After random assignment to experiment groups and prior to inoculation with either the mock of virus inocula, serum was collected from each pig. The mock and virus inoculated pigs were clinically scored daily from 0 to 14dpi. Mock-infected animals are always examined first. Clinical signs are determined daily by determining clinical impression scores and daily dyspnea scores One mock and four PRRS virus inoculated pigs were euthanized at 6hpi, 12hpi, 1, 2, 4, 7, 14, 28, 43, 56, 70, 84, 98, 112 and 126dpi. At 98dpi, two mock and five PRRS virus inoculated pigs were euthanized and 6 virus inoculated pigs were euthanized at 126dpi. At necropsy 18 tissues [lung, thymus (Thy), palatine tonsil (PT), nicatating membrane (NicM), nasal turbinate (NasT), spleen (Spl), lingual tonsil (LT), liver, kidney, heart, brain, and the following lymph nodes: lateral retropharyngeal (LRLN), sternal (STLN), tracheobronical (TBLN), inguinal (IngLN), medial iliac (MILN), mandibular (MDLN) and mesenteric (MesLN)] where collected for VI, nested RT-PCR, IHC and histopathology. Sera was also collected for virus isolation, nested RT-PCR and for detection of antibodies. For quasispecies analysis, lung, inguinal lymph node and tonsil were prepared for nested RT-PCR as previously described (Rowland et al, 1999, Virology 259:262-266) and products were either sequenced directly or cloned into TOPO vectors as described above.

VI. Results: *A. Targeted sites of acute and persistent PRRS virus replication and detection of virus, viral antigen and viral RNA from 6 hpi to 126 dpi.* PRRS virus strain 92-23983SD caused mild clinical disease characterized by rough hair coats, mild respiratory disease with occasional dypsnea, loose stools and eyelid edema in inoculated pigs. No clinical signs were seen in the mock inoculated pigs throughout the experiment. Gross lesions were not observed in either the mock or virus inoculated pigs at the time of euthanasia. Microscopically interstitial pneumonia (IP) was observed in acutely infected pigs from 2 to 14 dpi. Moderate IP was observed in 3/4, 1/4, 2/4 and 3/4 pigs at 2, 4, 7 and 14 dpi, respectively, with 1/4 pigs having marked IP at 14 dpi. Antibody responses were measured by ELISA and neutralizing assays. The ELISA S/P ratios peaked at 28 dpi, when acute infections began to subside, and then declined until 56 dpi, when there was a small increase in the ratio. Neutralizing antibodies were initially detected at 28 dpi and continued to increase until 112 dpi (Figure 1). All pigs were considered to be antibody positive by ELISA and neutralizing assays at the termination of the experiment. No antibody was detected in the mock-inoculated pigs.

The earliest viral replication was detected by RT-PCR at 6 hpi in the palatine tonsil of 1 pig. Virus was not detected in serum until 1 dpi and was present in most lymph nodes of pigs euthanized at 1 and 2 dpi. By 2dpi and especially 4 to 28 dpi virus was systematically detected in most tissue by either RT-PCR or VI (Table 1). Virus replication appears to peak at 4 to 14 dpi based on the increased numbers of virus isolations and demonstration of viral antigens in these tissues by IHC (IHC data not shown). There were only four positive VI results after 28 dpi and all were from tonsil or lymph nodes (Table 1). After 28dpi, the ability to detect infectious virus and viral antigens in any tissue drops precipitously as does the detection of viral nucleic acid in tissue other than tonsils and lymph nodes (Tables 1 and 2). Between 43 and 126 dpi the virus again becomes highly restricted to tonsil and lymphoid tissue and persists in these sites.
During acute infection defined as the time when infectious virus could be isolated from most tissues (up to 28 dpi), virus was most frequently ($p<.05$) isolated from tonsil, lymph nodes, lung, nicatating membrane, nasal turbinate, heart, and spleen (Table 2). After 28 dpi when infectious virus was difficult to isolate, viral RNA was detected by RT-PCR in tonsil, several lymph nodes (mandibular, inguinal, medial iliac) and lung irrespective of the time post inoculation. While there is a significant difference ($p<.05$) between the number of positive RT-PCR results with lingual tonsil compared to all other tissues, the palatine tonsil was just as reliable as the lingual tonsil for detection of PRRS viral RNA (Table 3, Figures 2 and 3). Various lymph nodes, lung, nicatating membrane and heart were also adequate clinical specimens for the detection of PRRS viral RNA from 6 hpi to 126 dpi. Overall, RT-PCR is the most reliable assay for detection of PRRS in both acute and persistent infection and is the only statistically significant technique for detection of PRRS virus from 43 to 126 dpi.

B. Viral quasispecies. Tissues (lung, inguinal lymph nodes and palatine tonsil) collected at 4, 7, 56, 70, and 126 dpi from three pigs at each time period were used for the detection of viral quasispecies. These tissues were used to generate both direct RT-PCR products from the tissue for sequencing to identify a consensus

sequence present in a particular tissue or pig. These sequences were compared to at least 8 clones from a single PCR reaction of each tissue to determine quasispecies diversity. The sequence data indicates that when ORF5 from pigs at 4 dpi is compared to pigs at 70 dpi there is an accumulation of mutations in the hypervariable region of this gene. These mutations principally consist of the substitution of an A for G at nucleotide 97 on ORF5. This sequence change was the most common change in 63% of the clones. The nucleotide change resulted in the substitution of a serine for glycine at amino acid 33. In one sample the change at this amino acid was from asparagine to tyrosine. These changes were found in samples of lung and inguinal lymph node at 4 dpi through 70 dpi . At 56 dpi, lung samples most frequently had substituted isoleucine for threonine at amino acid 26. Similar changes were also found for inguinal lymph nodes at 126 dpi. To date tonsil has not yielded sufficient PCR product for analysis. Sequences from similar sample times were the same, irrespective of the tissue.

VII. Discussion: Studies in our laboratory and those of other investigators indicate that lymph nodes and tonsil tissues are a preferred site for persistence of the PRRS virus. In our study, it was shown that the virus establishes residency in these tissues very early in infection. Previously, it was out hypothesis that the PRRS virus only replicated in lymphoid tissue predominantly during the persistent phase of infection. The early association of virus with lymph nodes has not been previously described and indicates that this virus has a high affinity for cells of the macrophage lineage. Primary replication of this virus in various lymph nodes also helps explains the early and extended viremia that lead to systemic disease with this virus.

Diagnosis of PRRS virus in acute infections has relied primarily on the isolation of virus from lungs and the demonstration of interstitial pneumonia. In this study, there is good correlation between isolation of virus from lungs, lesions and demonstration of viral antigens by IHC for the first 28 dpi. However, statistical analysis indicated that tonsil and lymph node tissue were actually more likely to be virus isolation positive than lung. Once acute infection subsides, lymph nodes and tonsil are the best tissue samples for demonstrating viral RNA by RT-PCR from 43 to 126 dpi. Virus could rarely be isolated from tissues after 28 dpi. Thus, to increase the chances of either isolating infectious virus or detecting viral RNA in PRRS virus infected pigs, it is recommended that lymph nodes and tonsil tissue be used in conjunction with the conventional sample of lung.

As in our previous studies, we observed the emergence of a new PRRS viral population identified by a single nucleotide change in the ectodomain. The glycine to serine change at amino acid 33 was the most common mutation observed. This change was noted in acute and persistent infections. Interestingly, Rowland et al (1999. Virology 259:262) previously reported a mutation on amino acid 34 that resulted in a change in the glycosylation site on the ORF5 gene. This mutation was found only in lymphoid tissue from persistently infected pigs. Thus, it appears that the glycosylation site from amino acids 34-37 may be particularly susceptible to mutation. Whether this is the result of a positive or negative selection process induced by the immune response or host factors has not been determined. What becomes more important to producers from this study is that the early association of the PRRS virus with lymphoid cells indicates that the virus has the capacity to modulate the immune system very early in the infection process and this may explain why secondary infections are prominent in PRRS infected herds and provide a means for the virus to escape elimination by the immune system.

Thus, PRRS virus colonizes tonsil and lymph nodes early in infection and persists in these sites for extended periods of time, whereas the virus appears to be eliminated over time from non-lymphoid tissues. These results agree with and verify other reported studies on the pathogenesis and persistence of virus. The detection of PRRS virus in lingual tonsil is a novel finding, but the significance of this site of viral replication was not determined. The presence of infectious PRRS virus in palatine and lingual tonsil could provide a potential site for shedding of PRRS virus from the oral cavity during acute and persistent infections.

VIII. Lay Interpretation: What is the significance of these results for producers? First, the most likely primary site for replication of the PRRS virus is lymphoid tissue, either tonsil and/or lymph nodes. The early

colonization of lymphoid tissue and the persistence of PRRS virus in lymphoid tissue indicate that PRRS virus has the potential to modulate the immune response early in infection. The persistence of PRRS virus in lymphoid tissues and tonsil provides a mechanism for shedding of this virus in bodily secretions such as saliva.

The PRRS virus can be detected in lymph nodes, tonsil, lung and other tissues during an acute infection virus isolation, IHC and RT-PCR. Overall, RT-PCR is the most reliable technique for detection of the presence of PRRS virus, because the viral nucleic acid can be consistently detected in both acute and persistent infections. Diagnostic laboratories should include tonsil and lymph nodes as part of the diagnostic sampling scheme in addition to lung. Since it is difficult to determine at what stage of infection pigs may be infected, the inclusion of tonsils and lymph nodes and RT-PCR in the diagnostic repertoire will markedly improve the chances of a positive diagnosis.

Finally, the results of this study indicate that variability in the ectodomain of ORF5 occurs in acute and persistent infections. It is possible that a particular quasispecies colonizes the pig early in infection and mutations accumulate over time in the ectodomain of ORF5. The mechanism of how PRRS established persistence remains to be determined, but the virus does change over time within individual pigs.

For additional information please contact David A. Benfield, at benfield.22osu.edu or phone 330-263-3703.

Publications:

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Waldner D, Fairbanks K, Holler L, Nelson C, Johnson C, Chase C, Christopher-Hennings J and Benfield D. 2001. Porcine reproductive and respiratory syndrome virus (PRRSV) in breeding swine: use of PCR on tonsil biopsies and sentinel pigs to detect viral nucleic acid and shedding in persistently infected pigs. 44th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, Nov 2-5, Hershey, PA, p. 61.

Waldner D, Fairbanks K, Holler L, Nelson C, Johnson C, Chase C, Christopher-Hennings J and Benfield D. 2001. Porcine reproductive and respiratory syndrome virus (PRRSV) in breeding swine: use of PCR on tonsil biopsies and sentinel pigs to detect viral nucleic acid and shedding in persistently infected pigs. Proceedings of the 82nd Meeting of the Conference of Research Workers in Animal Diseases, Nov 11-13, St. Louis, MO. Abstract 170.

Waldner D, Zeman D, Kasuke A, Ropp S, Arndt B, Nelson E, Fang Y and Benfield D. 2003. Temporal studies on the replication of PRRS virus in conventional pigs from six hours to 126 days post-inoculation. 46th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, Oct 11-13, San Diego, CA p. 141.

Waldner D, Zeman D, Kasuke A, Ropp S, Arndt B, Nelson E, and Benfield D. 2003. Lymphoid tissues may represent not only sites of persistence but early sites of replication of porcine reproductive and respiratory syndrome (PRRS) virus. Proceedings of the 84th Meeting of the Conference of Research Workers in Animal Diseases, Nov 9-11 Chicago, IL. Abstract 117P.

Table 1. Number of pigs with tissues positive by nRT-PCR and (virus isolation) from 6 hours to 126 days post-inoculation (PI)																				
Time PI	**No. of Pigs with Positive Tissue**	**LT**	**PT**	**IngLN**	**MILN**	**NicM**	**MDLN**	**STLN**	**LRLN**	**Heart**	**Lung**	**TBLN**	**Thy**	**NasT**	**MesLN**	**Kidney**	**Spl**	**Brain**	**Liver**	**Total**
6hpi	1 (0)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (0)
12hpi	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0)
1dpi	2 (2)	1 (1)	0	1	1	0	1	0	1	1	1 (1)	1 (1)	1	2	1	0	0	0	0	12 (3)
2dpi	3 (1)	3 (1)	2 (1)	2 (1)	1 (1)	3 (1)	1 (1)	1 (1)	1 (1)	1	1	0	1 (1)	2 (1)	1 (1)	0	1 (1)	0	0	21 (12)
4dpi	4 (4)	2 (2)	4 (3)	4 (2)	4 (1)	2	2 (2)	3 (4)	4 (4)	3	4 (2)	4 (1)	3 (3)	4 (4)	3 (2)	1 (1)	2 (1)	2	2	53 (32)
7dpi	4 (4)	4 (3)	4 (4)	4 (4)	4 (4)	4 (3)	4 (4)	3 (4)	3 (4)	3 (2)	4 (4)	4 (4)	3 (3)	4 (4)	3 (3)	1 (3)	3 (4)	3 (1)	1	59 (58)
14dpi	3 (4)	3 (3)	3 (4)	2 (4)	3 (4)	3 (2)	3 (3)	3 (3)	3 (4)	3	3 (3)	3 (3)	1 (1)	3 (2)	3 (2)	2 (3)	2 (3)	0	0 (1)	43 (44)
28dpi	4 (4)	4 (2)	4 (3)	3 (2)	3 (2)	4	3 (2)	3 (2)	3 (2)	3 (1)	4 (3)	3 (4)	1 (1)	3 (1)	1	0	0	0	0	42 (25)
43dpi	4 (1)	3	2 (1)	1	2	2	1	1	2	0	2	0	1	0	0	0	0	0	0	17 (1)
56dpi	4 (2)	2	2	3 (1)	4 (1)	1	1	2	2	1	2	2	0	1	1	0	1	1	0	26 (2)
70dpi	4 (0)	3	4	2	0	0	3	2	1	1	2	1	1	0	0	1	0	1	0	22 (0)
84dpi	2 (1)	2	2 (1)	0	0	0	2	0	1	0	0	1	1	1	0	0	0	0	0	10 (1)
98dpi	4 (0)	1	3	4	2	0	1	2	0	1	0	0	0	0	0	0	0	0	0	14 (0)
112dpi	4 (0)	4	1	1	1	3	1	1	1	0	0	0	0	0	1	0	0	0	0	14 (0)
126dpi	5 (0)	5	2	1	1	3	0	1	0	NA	0	1	1	0	0	1	0	0	0	16 (0)

Numbers in parentheses represent number of pigs positive by virus isolation.
If number with parentheses is absent then tissue was virus isolation negative.

Table 2. Total number of positive virus isolation (VI) results by tissue type from 6 hpi to 126dpi.

Tissue	Number of pigs with positive VI from 6 hpi to 28 dpi[a]	Log Percent least square means of pigs with positive VI from 6 hpi to 126 dpi[b]	Significance (p<.05)[c]
Lingual tonsil	12	-1.04547444	a.b.c
Palatine tonsil	15	-0.83854468	a
Mandibular lymph nodes	12	-1.00073072	a,b,c
Inguinal lymph nodes	13	-0.94319431	a,b,c
Medial iliac lymph nodes	12	-0.94319431	a.b.c
Lung	13	-0.90898271	a,b
Nicatating membrane	6	-1.2071782	a,b,c,d
Lateral-retropharyngeal lymph node	15	-0.80456487	a
Sternal lymph nodes	12	-0.86210129	a
Nasal turbinates	12	-1.08182374	a,b,c
Tracheal bronchial lympn nodes	13	-0.99007573	d,e,f
Heart	3	-1.6603979	a,b,c
Mesenteric lymph nodes	8	-1.17754923	a,b,c,d
Thymus	9	-1.33552601	c.d.e
Spleen	9	-1.17754923	a,b,c,d
Brain	1	-2.33419191	f
Kidney	7	-1.32103102	d,e
Liver	1	-2.04431623	e,f

[a] Number of pigs from which virus was isolated from tissue between 6 hpi and 28 dpi. Virus was isolated at 43-70 dpi from the palatine tonsil of one pig, the inguinal lymph node of one pig and the medial iliac lymph node of one pig.

[b] Comparison of percent of positives/total number of samples by least squares means (p<.05).

[c] Similar letters indicate no significant difference between tissues.

Table 3. Total number of positive RT-PCR results by tissue type from 6 hpi to 126dpi.

Tissue	Number of pigs with Positive RT-PCR From 6 hpi to 28 dpi	Number of pigs with positive RT-PCR from 43 to 70 dpi	Number of pigs with positive RT-PCR from 70 to 126 dpi	Percent least square means of pigs with positive RT-PCR from 6 hpi to 126 dpi[a]	Significance (p<.05)[b]
Lingual tonsil	17	8	12	0.68	a
Palatine tonsil	18	10	8	0.59	a,b
Mandibular lymph nodes	14	5	4	0.52	b,c
Inguinal lymph nodes	16	6	6	0.46	b,c
Medial iliac lymph nodes	16	6	4	0.46	b,c
Lung	17	6	0	0.42	c,d
Nicatating membrane	16	3	6	0.41	c,d
Lateral-retropharyngeal lymph node	14	5	2	0.41	c,d
Sternal lymph nodes	13	5	4	0.36	c,d
Nasal turbinate	18	1	1	0.36	c,d
Tracheal bronchial lymph nodes	15	3	2	0.36	c,d
Heart	13	2	1	0.36	c,d
Mesenteric lymph nodes	12	1	1	0.28	d,e
Thymus	10	3	1	0.28	e,f
Spleen	8	1	0	0.17	e,f
Brain	5	2	0	0.12	f
Kidney	5	1	1	0.11	f
Liver	3	0	0	0.05	f

[a] Total number of pigs with positive RT-PCR tissue sample from 6 hpi to126 dpi.

[b] Comparison of percent of positives/total number of samples by least squares means (p<.05). Similar letters indicate no significant difference between tissues.

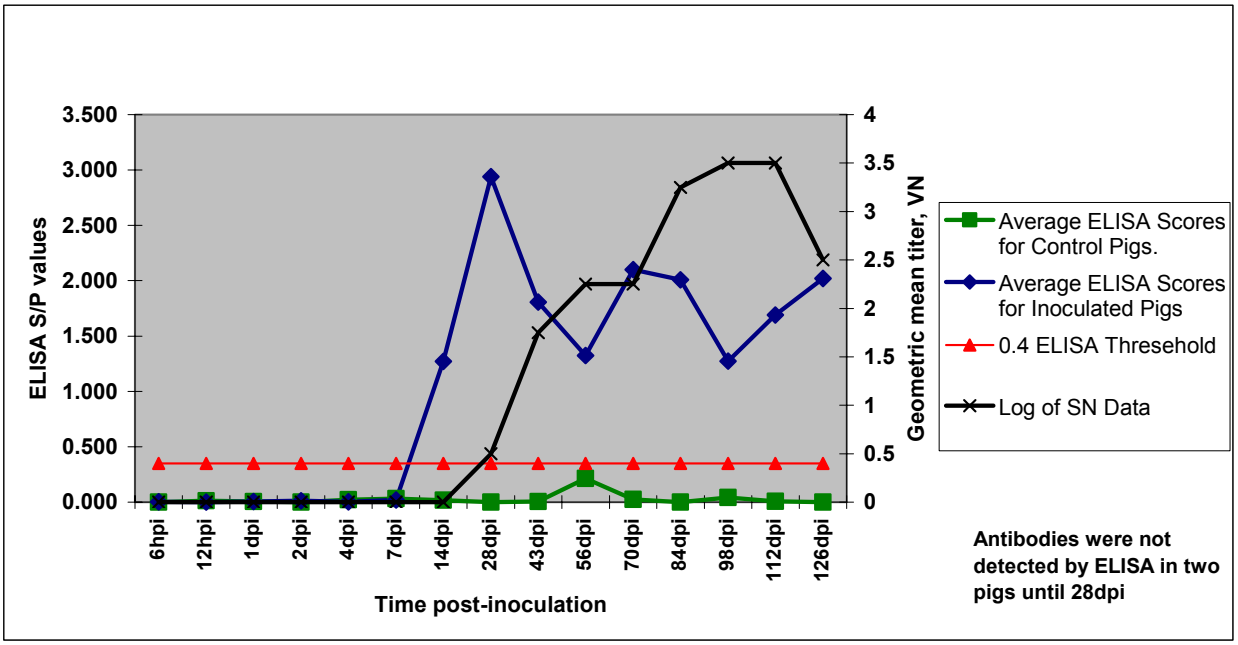

Figure 1. ELISA S/P ratios and virus neutralization titers (VN) in inoculated and mock-inoculated pigs from 0 to 125 dpi.

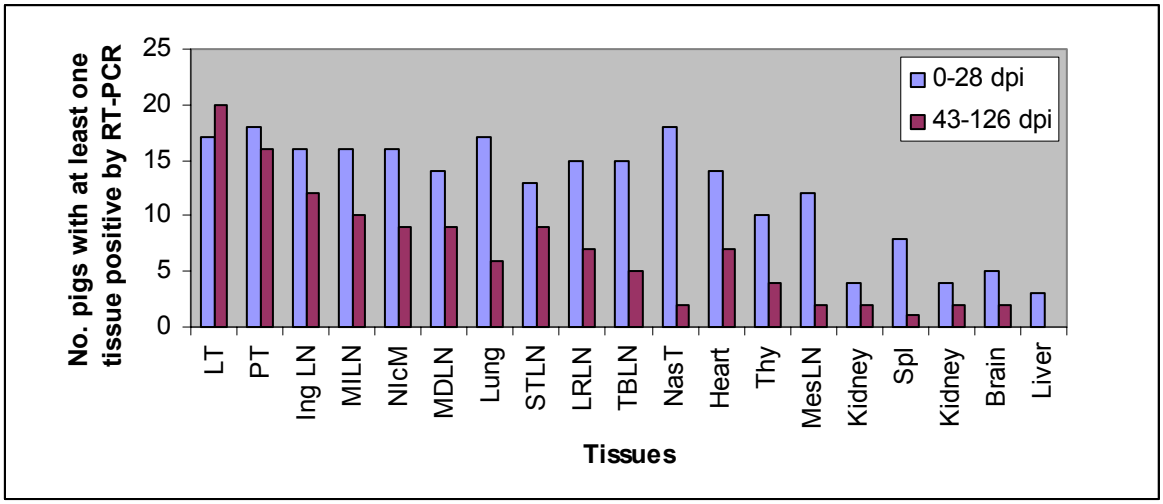

Figure 2. Number of pigs with at least one tissue positive by RT-PCR during either acute (0-28 dpi) and persistent infection (43-126 dpi).

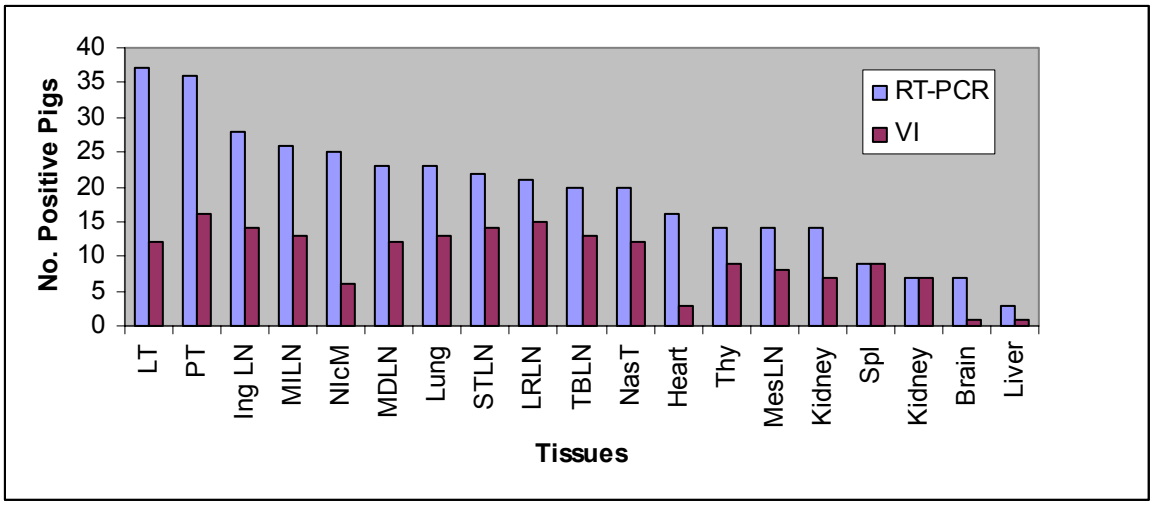

Figure 3. No. of total tissues positive by tissue type using nested RT-PCR and virus isolation (VI) irrespective of hours or days post-inoculation.