RESEARCH REPORT
SWINE HEALTH

Title: Characterization of the Fetal Immune Response to PRRS - **NPB # 01-059**

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Abstract

Inoculation of 50-day fetuses with attenuated PRRSV (NADC-8:251 passages) that does not typically cause abortion, results in a >100-fold increase in serum IgG three weeks later. This suggested that polyclonal B cell activation could be part of the fetal pathology. Since fetal studies do not allow the same animal to be progressively studied and such studies were criticized because wild-type PRRSV was not used, germfree (GF) isolator piglets became the major focus of the second half of the study.

We show that inoculation of GF or colonized isolator piglets with wild-type PRRSV results in a polyclonal lymphoproliferative disorder characterized by lymph node hypaplasia, >300-fold elevation of IgG levels, apparent circulating immune complexes, autoantibodies to Goli apparatus and dsDNA and damage to the kidney including deposition of IgG in the glomerulus (10). We speculate the piglets immune system is diverted away from pathways that allow complete viral clearance thus generating carrier animals.

Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) remains a serious problem to the swine industry. Immunological studies on PRRSV have so far been superficial and often difficult to interpret because of the many uncontrollable environmental and maternal factors simultaneously acting on the immune system. Since PRRSV is able to cross the placenta, the effect of the virus on the immune system can be studied in a controlled environment. Moreover, understanding the fetal response to PRRSV (or the lack of one) may help explain PRRSV-induced abortion or the failure of piglets exposed as fetuses, to completely

clear the virus after birth. Nevertheless the "fetal model" has several disadvantages. First, animals can only be sampled once after inoculation (at necropsy). Second, the danger exists for virus to spread among the litter so that all fetuses in any one gilt must be treated the same way. This greatly increases the number of gilts needed. Third, only attenuated virus can be used *in utero* raising the criticism that data obtained with attenuated PRRSV may be artifactual when compared to wild-type virus. Therefore we wanted to confirm that any observation made in the first portion of the research conducted on fetuses could be confirmed by using newborn GF piglets reared in isolation and inoculated with wild-type PRRSV. Furthermore, this design requires fewer animals and allows periodic sample collection from the same animal.

Objectives

The original project objectives were to test the following two **hypotheses** using an attenuated strain of the PRRSV in fetal piglet. We believed this could serve as a model to study the immunobiology of the fetal infection without the complications of the lethal effects of the wild-type virus when used to inoculate fetuses.

1. The vigorous late term antibody response of fetal piglets to viral inoculation on day 50 is the consequence of latent development of fetal immunocompetence to the PRRSV.

2. Replication of the PRRSV prior to 80 days of gestation is confined to a remote fetal site in which the number of susceptible cells is limited thus limiting exposure to the immune system.

During the course of the study and after having completed several fetal studies, we added two additional objectives that we believed would eventually be of greater value in resolving the PRRS pandemic.

3. Determine if the apparent polyclonal B cell activation seen in fetuses when attenuated PRRSV was used could be duplicated after birth using wild-type virus.

4. Monitor the kinetics of the clinical response on a daily basis and the serological response on a weekly basis.

Materials and Methods

1. Laboratory, inoculation and recovery of fetuses. All animal studies were conducted at the NADC, a AALAC certified laboratory. Between 48 and 50 days of gestation pregnant gilts underwent surgery for *in utero* inoculation of all fetuses with either virus or a virus-free sham inoculation (Table 1). Gilts were allowed to recover and housed in isolation facilities until time for necropsy. Virus inoculum (0.2 of $1x10^4$ $CDDID_{50}$) consisted of a cell culture of attenuated PRRSV strain (NADC-8) or a sham inoculum consisting of 0.2 ml PRRSV-free cell culture lysate prepared in similar fashion. Gilts and piglets were euthanized with an intravenous administration of pentobarbital.

2. Experimental design. Table 1 is a modification of the original table that described the actual animal studies preformed.

TABLE 1
Influence of Inoculation Age on the Fetal Immune Response

No. Gilts[a]	Age at Inoculation	Age of Fetus at Recovery			
		60	70	90	110
16	50	0	5	6	5
6	70	N.A.	N.A.	3	3
4	90	N.A.	N.A.	N.A.	N.A.

[a] This includes piglet inoculated with attenuated PRRSV and the sham inoculant. Two-thirds of the animals received virus, the remainder received the sham.

3. Collection of fetal tissues. The fetuses of the euthanized gilts from the experiments in Table 1 were carefully removed from the uterus and as shown in Table 2, various tissues collected.

4. Virus isolation and PCR assays for PRRSV. Virus isolation and PCR assays for detection of PRRSV were conducted at the NADC as previously described (Table 2 footnote).

TABLE 2
Collection of Various Fetal Tissues for Measuring Different Parameters

Tissue	Virus Isolation and viral PCR (1,2)	Ig & Ab Det'n[a]	VDJ usage[b]
Blood (serum + cells)	+	+	+
Spleen	+	-	+
Thymus	+	-	+
MLN	+	-	+
Partoid gland	+	-	+
IPP	+	-	+

[a]See V-5,6
[b]See V-7

5. Immunoglobulin (Ig) and PRRSV-specific antibody responses. Fetal and neonatal serum IgG, IgA and IgM levels were determined by sandwich ELISA as previously described (3) and using single radial diffusion in 1% agar gels (4). Anti-PRRSV ELISA activity was measured using the IDEXX assay.

6. Western blot analysis. Wild-type and attenuated PRRSV were treated with denaturing buffer, the constituent proteins separated by SDS-PAGE and blotted onto PVDF. PRRSV-specific antibodies were detected using sera from a hyperimmunization swine followed by goat anti-swine IgG conjugated to HRP.

7. Spectratyping. Rearranged VDJs were recovered by RT-PCR. A third round PCR was used to amplify the CDR3 region which was then spectratyped on 6% polyacylamide gel as previously described (5).

8. Isolator piglets. Caesarian surgery and the rearing of piglets in isolator units was carried out by standard practices used at the NADC. These were done by Dr. Rodger Spaete. Colonization was done as previously described (6) using *E. coli* G58-1 kindly provided by Dr. David Francis, South Dakota State University.

9. Fast performance liquid chromatography (FPLC). FPLC was performed on a Superose 6 column (Pharmacia, Piscataway, NJ) using a Waters System HPLC unit.

10. Anti-nuclear antibodies. Autoantibodies of the IgG isotype were detected in piglet serum using the Immuno Concepts HEp-2 Fluorescent ANA Test System (Immuno Concepts, Sacramento, CA). IgG anti-dsDNA was detected using the INOVA QUANTA Lite ds DNA ELISA kit (INOVA Diagnostics, Inc., San Diego, CA).

Results

Figure 1 summarizes data on IgG and IgM in the sera of 119 fetal piglets inoculated with either attenuated PRRSV or SHAM at different times during gestation. As we previously showed (7) older fetuses have higher Ig levels but those fetuses sampled at 110 DG that were inoculated on DG50 have mean IgG levels that are only 3-fold above controls whereas 70 DG fetuses inoculated on DG50, have IgG levels that are >100 fold higher than SHAM controls (Fig. 1). This pattern is consistent with data presented below on infected, isolator piglets. In these studies, IgG levels become elevated 300-fold just 3 weeks after inoculation (Fig. 2). This might hint that the polyclonal activation that characterizes other arterivirus infections (8; see below) also occurs with PRRSV and the fetuses respond in the same manner to attenuated PRRSV as isolator piglets respond to wild-type PRRSV.

A surprising observation in the current study emerged at first as a technical problem but became as interesting immunobiological characteristic of PRRSV infections. The original intent was to calculate values for Specific Activity (see Materials and Methods) to determine if PRRSV caused polyclonal activation. However, we observed that the sera from infected fetuses did not titrate with the proper slope in Specific Antibody immunoassays. This lead to the discovery that IgG and IgM in PRRSV infected animals (fetal, neonatal or adult; Table 3) readily bind to polystyrene, confirming the observation by Cafruny et al. (8) for mouse LDV, another arterivirus. Thus, 80-90% of the apparent "PRRSV-specific antibodies" can be attributed to those binding to plastic (Fig. 3). Therefore we suspect that the IDEXX test simultaneously measures PRRSV-specific IgG plus non-antibody IgG in PRRSV-infected fetuses that binds plastic. Cafruny et al. (8) believed this resulted from the propensity of circulating immune complexes to spontaneously bind to plastic even in the presence of non-ionic detergent. However, the IDEXX test most likely also detect anti-PRRSV antibodies

since these are detectable by Western blot using the serum of a convalescent adult animal and the sera of a PCOL isolator piglets that have very high serum IgG levels.

TABLE 3

Representative results for three different litters[a] of piglets

	3 weeks				6 weeks				9 weeks			
	Virus[b]		IDEXX[c]		Virus		IDEXX		Virus		IDEXX	
	PRRSV	Sham	PRRSV	Sham	PRRSV	Sham	PRRSV	Sham	PRRSV	Sham	PRRSV	Sham
A	0/10[d]	0/8	0/10	0/8	3/8	0/6	0/8	0/6	13/13	7/11	11/13	1/11
B	5/12		0/12		11/24		0/24		16/24		8/24	
C		0/15		0/15		0/14		0/14		0/13		0/13

[a]Litters exposed to an attenuated PRRSV strain via amniotic injection at or about gestation day 50 (DG50). In some litters (row A) all fetuses in one uterine horn were exposed to the virus inoculum (PRRSV) and all fetuses in the contralateral horn were exposed to sham inoculum (Sham). In subsequent litters, all fetuses within the litter were exposed to either virus (row B) or sham (row C) inoculum. At about 3, 6, and 9 weeks post fetal exposure, the dam was euthanized and the litter recovered. Each fetus was tested for the presence of PRRSV and PRRSV-specific antibody using the IDEXX system.

[b]Proportion of fetuses positive by PRRSV virus isolation.

[c]Proportion of fetuses positive for PRRSV-specific antibody by IDEXX ELISA. Numerator equals number of virus or IDEXX positive fetuses and denominator equals number of fetuses tested.

TABLE 4

Serological and virological characteristic of fetuses in litter 18

				OD 405 cm at indicated dilution[d]			
Fetus tested[a]	PRRSV[b] tested	IDEXX[c]	Western blot	Dilution	PRRSV	Plastic	Diff
Reference Standard	+	?	+/+	1:2000	0.902	0.316	0.586
18L1	+	1.857	-/+	1:10	0.268	0.299	0.039
18L2	-	0.317	?	1:10	0.282	0.170	0.112
18L3	-	0.706	?	1:10	0.236	0.124	0.112
18L4	-/+	0.881	?	1:10	0.247	0.141	0.106
18R1	+	2.746	?	1:10	0.241	0.131	0.110
18R2	+	0.698	?	1:10	0.769	0.832	-0.063
18R3	-	0	?	1:10	0.175	0.1320	0.045
18R4	+	0.115	?	1:10	0.035	0.031	0.004

[a] Fetuses in litter 18 were inoculated at DG50 and sampled at DG110.

[b] Determined by viral recovery and PCR.

[c] Determined according to the manufacturers recommendations. Values above 0.4, are considered positive. The Reference Standard was not tested by IDEXX (?).

[d] Microtiter plates were treated with PBS, pH 7.0 or PRRSV in PBS overnight at RT. The wells were washed and blocked with casein blocker (Pierce Chemicals, Rockford, IL) and then incubated with swine serum in PBS-Tween. Detection was done using rabbit anti-γ chain followed by goat anti-rabbit conjugated to alkaline phosphatase. The detection system did not detect blocker, plastic or PRRSV. Diff = different in OD405 which is not proof of PRRSV-specific antibodies.

The studies using isolator piglets and wild-type PRRSV (NADC-8) has provided some valuable insight into immunomodulation by PRRSV. The striking elevation of Ig levels shortly after inoculation suggests that this arterivirus is a very powerful activator of B-cells (Fig. 2). Spectratypic analysis now confirms this to be polyclonal (Fig. 4). In humans, polyclonal activation is characteristic of some autoimmune diseases and autoantibodies were reported in LDV infections in mice (8).

In addition to polyclonal (or monoclonal) activation, we have presented evidence for antigen-driven antibody repertoire diversification in PRRSV-infected fetuses (Table 5). Infected animals have increased levels of somatic hypermutation and shifts in V_H usage that is characteristic of diversification (Table 5).

Table 5
Sequence Analysis of Thymic VDJ Transcripts (7)

Transcript Source	Number Examined	CDR1	CDR2	CDR3	Total Mutations	$\frac{V_HA + V_HC + V_HX^{b}}{V_HB + V_HE}$
Normal IgM	17	0.019	0.014	0.010	0.015	0.94
Normal IgA	15	0.067✦	0.037ΔΔ	0.008✦	0.034ΔΔ	0.92
Normal IgG	12	0.039✦	0.020✦	0.000✦	0.020Δ	3.8
PRRS IgM •	13	0.011^{ns}	0.022**	0.084**	0.033	5.5
PRRS IgA •	19	0.046^{ns}Δ	0.046**ΔΔ	0.101**✦	0.057ΔΔ	1.7
PRRS IgG •	15	0.057^{ns}ΔΔ	0.051**ΔΔ	0.120**✦	0.062ΔΔ	5.0
6-wk germfree♦	19	0.012^{ns}	0.017^{ns}	0.023^{ns}	0.013	0.5
6-week colonized♦	21	0.081**	0.076*	0.058*	0.076	13.0
Adult ♦	34	0.144**	0.186**	0.062*	0.156	(8.5)

[a] Mutations/nucleotide. In the case of CDR3, mutation/nucleotide could only be recognized in D_H and the J_H sequence 5' of the invariant tryptophan codon. Student's t used to test the hypothesis that the mutation frequency in transcripts from PRRS-infected, germfree, colonized and adult differ from thymic transcripts in normal fetuses. ** significantly different from normal transcript at 0.01 level; * significantly different at 0.05 level; [ns] non-significant. Statistical comparisons to previously published transcripts not separated according to isotype were done against a pooled average of IgM + IgA + IgG transcripts.

A second statistical analysis was undertaken to compare the mutation frequency in switched isotypes (IgA & IgG) compared to that in IgM transcripts from the same animal group. ✦ = not significantly different from IgM transctipts; Δ = significantly different at the 0.05 level; significantly different at the 0.01 level.

[b] Usage of V_HC and V_HA together with seldom used genes like V_HL or V_HK characterizes differentiation of the porcine V_H repertoire while usage of especially V_HB (analogous to V_H81 x in mice) and to a lesser extend V_HE, reflect the undiversified repertoire. V_HX includes V_H genes such as V_HL and V_HK that are periodically used in diversified repertoires. Thus the ratio of V_H usage is a useful parameter of repertoire diversification.

• Data from 95 day fetuses infected at 50-days *in utero* with Porcine Respiratory and Reproductive Syndrome (PRRS) virus.

♦ Data calculated from previously published sequences obtained from various non-thymic sites. The frequency of somatic mutation in the CDR3 region of adult sequences is underestimated because of the difficulty in distinguishing highly mutated D_H segments from nucleotide additions.

With regard to Objective 1, the results provide little information about functional immune competence because the modest immune response (VN and IDEXX) is overshadowed by massive increases in especially IgG. Thus, any specific response could simply be the result of polyclonal stimulation of the pre-immune repertoire, not a response driven by viral antigens. Thus, we appropriately shifted our focus to the dominant phenomenon - - polyclonal activation. Objective 2, was not pursued because of time and material constraints and the logical shift to Objectives 3 and 4. The question raised in Objective 2 is still intriguing and needs eventually to be addressed. The magnitude of the effect of PRRSV may parallel the development of the immune system.

Our decision to shift some of our objectives (IV. Objectives, above) was discussed in the INTRODUCTION. Simply stated, we wanted to reach the heart of the problem and obtain data worthy of publication in a high visibility immunology journal. Figure 2 clearly shows the kinetics of the response to wild-type PRRSV as well as the magnitude of the effect. Furthermore it shows that the 3 week effect seen in fetuses inoculated with attenuated PRRSV (Fig. 1) is not an artifact of using an attenuated virus.

The use of 12 isolator piglets has allowed much more information to be obtained than could have been done using 100 fetuses. In addition to the elevated polyclonal Ig response (Figs. 2 & 4), we showed by using FPLC that the sera of PRRSV-infected isolator piglets contain high molecular weight IgG and autoantibodies to Golgi and dsDNA (9, 10). We also show in the same presentation (9) and publication (10) that the submandibular and bronchial-associated lymph nodes are 5-10 fold larger than normal and that the kidney is characterized by distended glomeruli and mononuclear infiltrates. Using immunohistochemistry, glomeruli contains "lumpy-bumpy" IgG deposits. All of these observations are consistent with the view that infection of pigs with PRRSV results in a lympho proliferative disorder associated with autoantibodies, immune complexes and kidney damage.

VII. Discussion

Our studies are the first to identify a major component of PRRSV-induced immunopathology (10). This should not have been a surprise since studies with the mouse artivirus LDV have shown many of the same features. What is surprising is that it took >15 years for someone to identify that the same immunopathology is associated with PRRSV infections given the copius volume of literature available.

Identifying the phenomenon is only the initial step in seeking a remedy to the pandemic. Two studies are critical. First, to determine how this immune dysfunction disables the immune system's ability to eliminate the virus. In a recent collaborative study (11) we showed that FMDV infection down-regulates the Th1 response and the production of Interferon γ which may prevent total elimination of the virus. Since PRRSV results in B cell hyper proliferation (a Th2 event) it is plausible that the PRRSV survives total elimination through down regulation of cytotoxic and inflammation T cell response. This could allow persistence of an infected carrier state in which anti-viral antibodies have disappeared.

The second key experiment is to determine how PRRSV causes this B cell proliferation. Is it a B cell superantigen, misdirection of cytokine regulation or a T cell superantigen capable of indirectly causing both phenomena?

With this information available, it should be possible to engineer a vaccine that does not cause negative B cell proliferation but rather stimulates a Th1 or cytotoxic response that can lead to total viral elimination. This is plausible since there is no evidence that swine are unresponsive to PRRSV antigens only that the response may be misdirected.

VIII. Lay Interpretation

PRRSV causes abnormal development of immune function. This is believed to allow the virus to persist in carrier animals that remain relatively healthy. Such disregulation of the immune is a common feature of many viral infections. Good parasites exist by only partially disabling their host's immune system which in turn allows them to survive and be transmitted without causing such wide-spread damage that the host species is eliminated. Until a thoroughly dependable vaccine is developed through genetic engineering, detection of the carrier status of animals is likely to be an important safeguard that can be used in connection with proper management practice. Whether the carrier state can be reliably detected by assays that depend on serum antibodies in animals that have recovered from a hyperplastic condition, needs to be addressed. [J. E. Butler, The University of Iowa; john-butler@uiowa.edu]

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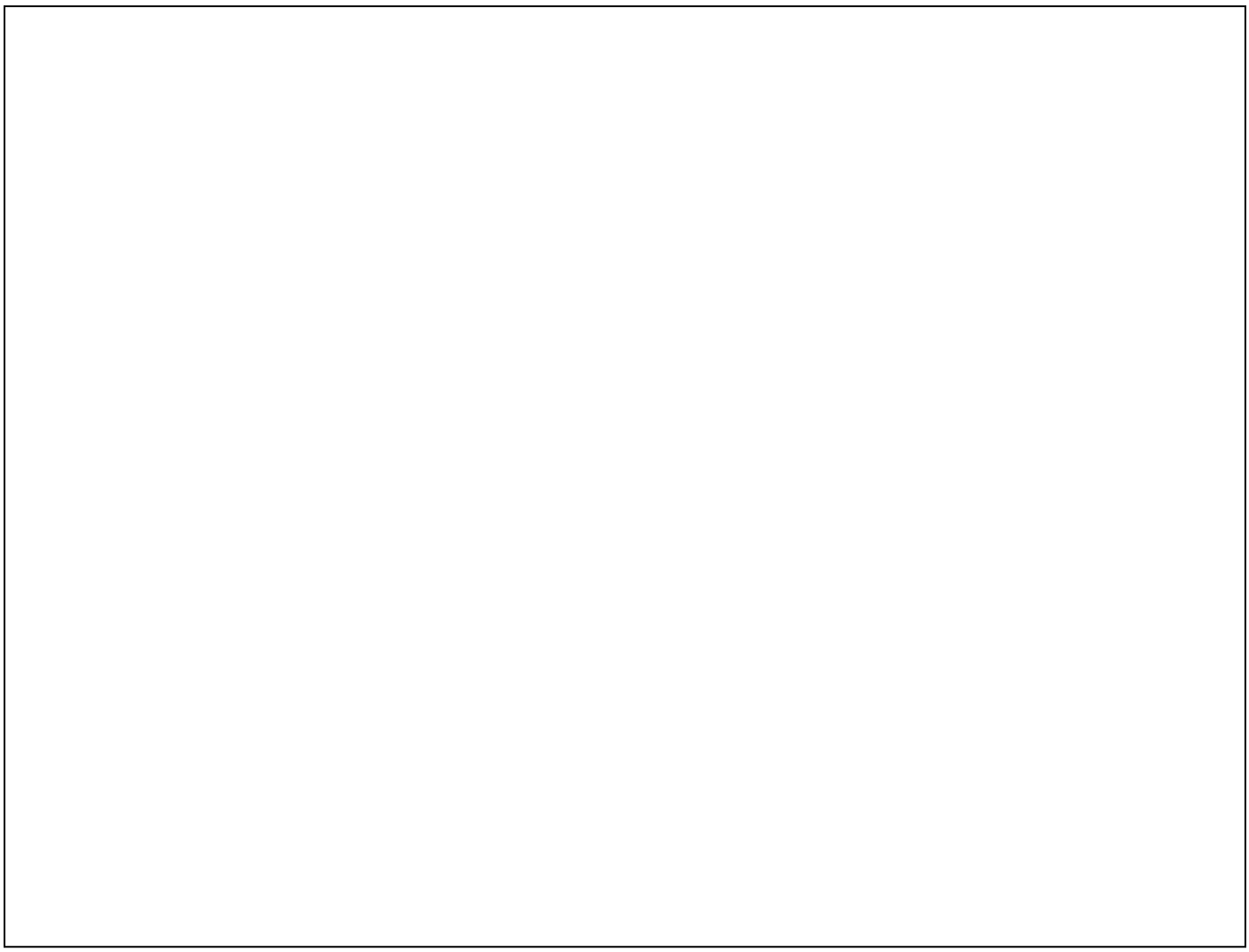

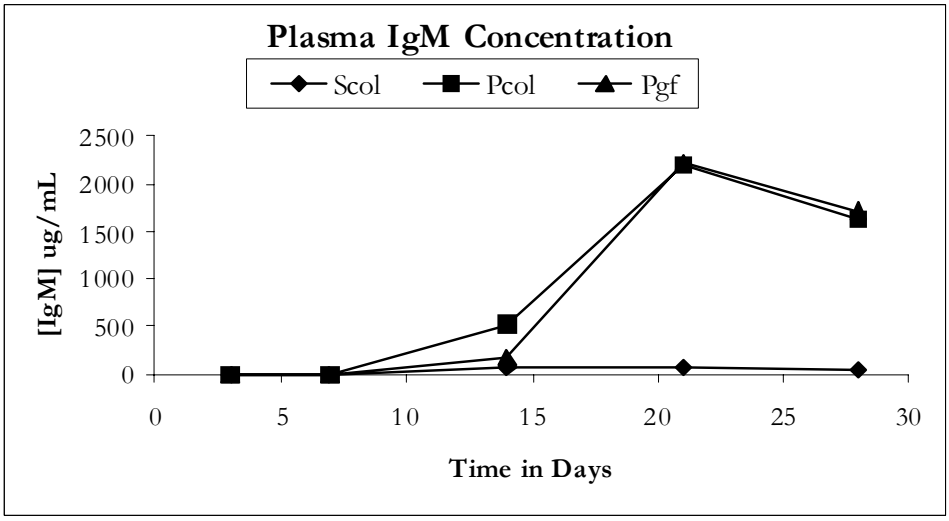

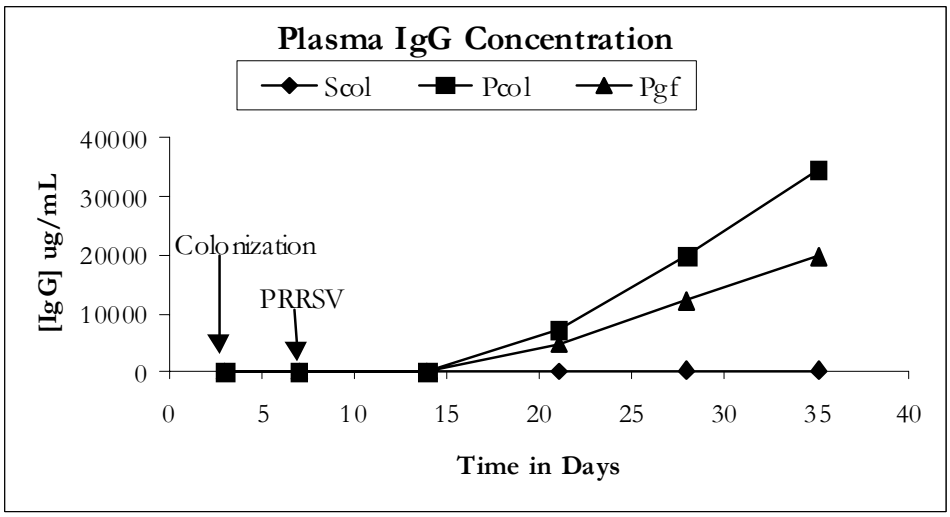

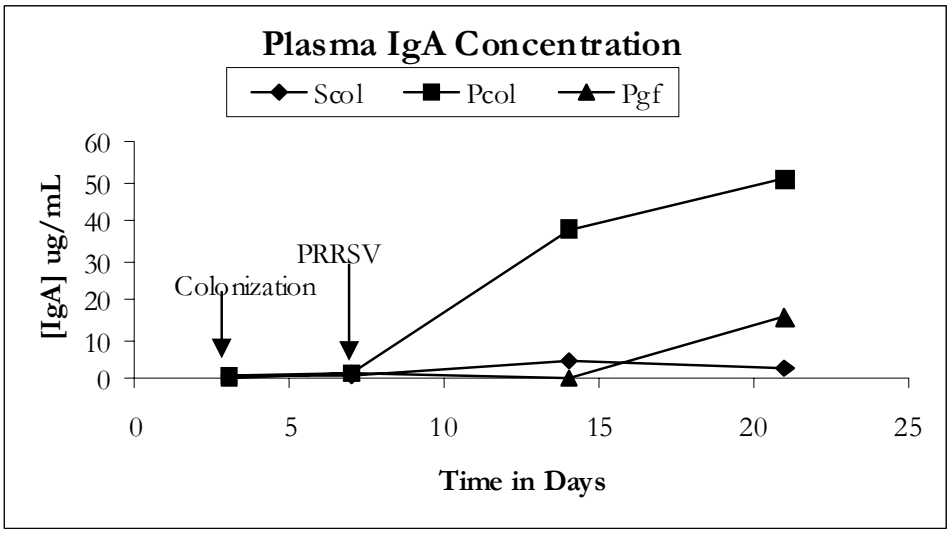

Figure 2. Immunoglobulin levels in the sera of PRRSV-inoculated, colonized isolator piglets (Pcol), inoculated germfree piglets (Pgf) and sham inoculated colonized controls. Error bars omitted to reduce clutter

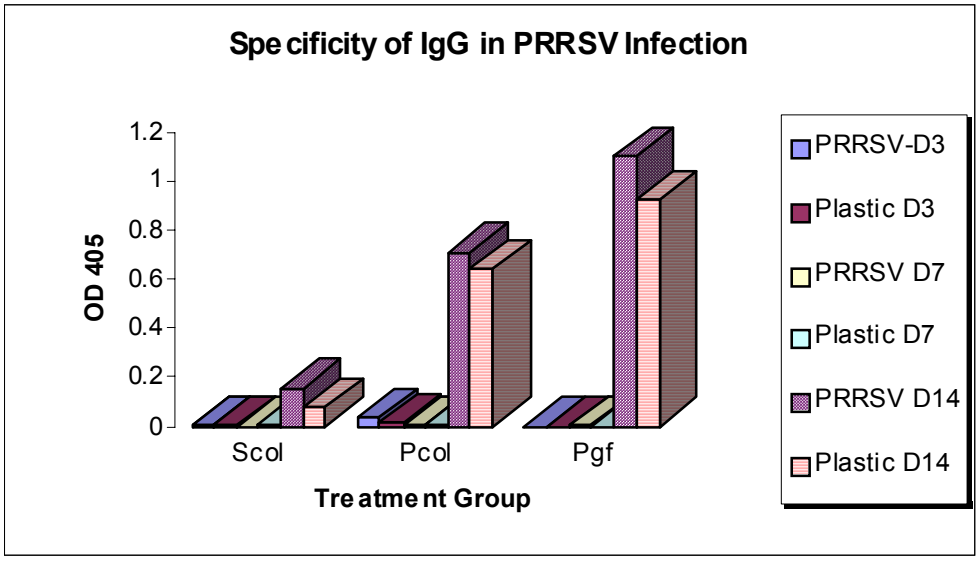

Figure 3. ELISA "titers" measured at a fixed dilution in different treatment groups. Sera were tested for specificity to immobilized PRRVS after blocking with casein "Superbloc" (Pierce Chemicals) and to microtiter wells treated in the same manner but lacking the PRRSV. D=day; Treatment groups are the same as those described in Figure 1.

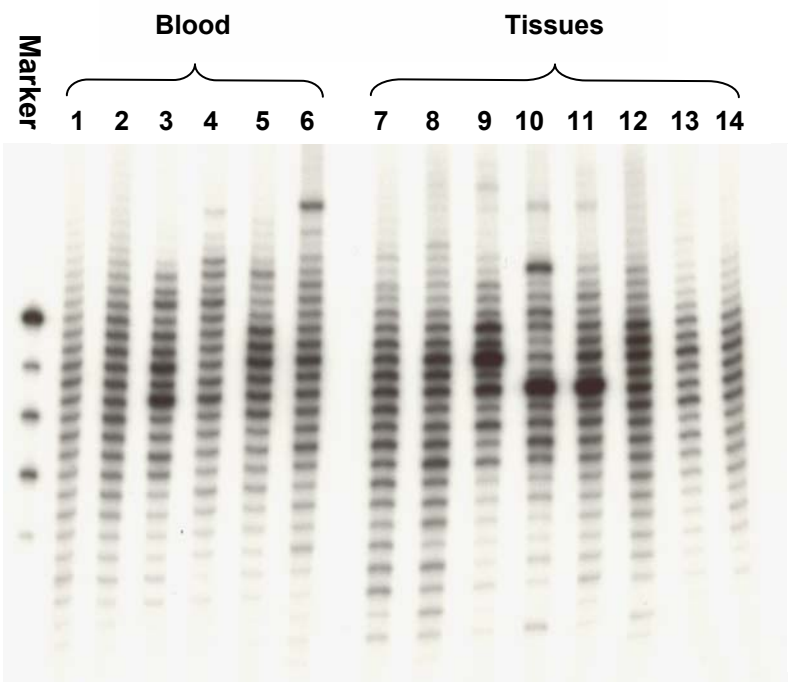

FIGURE 4. Blood and tissue B-cell clonality. Spectratypic analysis of the CDR3 lengths in a representative population of B-cells from SCOL, PCOL and PGF piglets. *Lanes 1-2,* SCOL piglets 1 & 2; *lanes 3-4,* PCOL piglets 1 & 2; *lanes 5-6,* PGF piglets 1 & 2. *Lanes 7-8,* SCOL piglet 2 MLN & BLN; *lanes 9-11,* PCOL piglet 1 MLN, Tonsil, BLN, respectively; *lanes 12-14,* PGF piglet 1 MLN, Tonsil, BLN, respectively. Marker indicates reference standard CDR3