RESEARCH REPORT
SWINE HEALTH

Title : Role of Cachectic Cytokines in PMWS **- NPB #: 06-074**

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Industry Summary: An opportunity was presented whereas sequential samples were collected in the midst of a natural outbreak of PMWS. This provided the basis to evaluate immunological and virological events in the midst of an outbreak.

With this natural outbreak and the required samples we could test questions related to whether the cachexia in PMWS was due to the induction of inflammatory cytokines PCV virus load Results revealed an increase in TN-alpha across groups, an increase in PCV load across groups and a correlation of TNF and PCV virus load in a subset subjected to regression analyses. It should be noted that the cytokine levels and virus load were performed on banked samples, prohibiting additional sample collection.

Scientific Abstract: The inflammatory mediators TNF-alpha,and IL-1 along with PCV-2 viral load were determined from pigs experiencing a high mortality due to a natural outbreak of PMWS. The objectives were to test the hypothesis that PMWS results from an immunological triggering event which produces a strong immune response which in turns drives virus load. There was no correlation between TNF-alpha levels and PCV-2 log copy number at 0, 3, 14, and 30 days post introduction for naïve piglets introduced in the monitor group at 37 days post inoculation (P value >0.05). There was an association between levels of TNF-alpha and PCV-2 load among PCV-2 viremic pigs at 0dpi (coefficient correlation: 0.67, P≤0.05); 3dpi (coefficient correlation: 0.83, P≤0.05). At 14dpi and 30dpi coefficient correlation are reported as 0.37 and 0.49

respectively. Due to testing on banked samples and low sample size, major conclusions need to be guarded. As in most studies additional work is clearly needed to sort out the pathophysiology associated with PMWS.

I. Introduction: PMWS has proven very difficult to control and treat in the field. This is partly due to a poor understanding of the pathogenesis of this disease. Moreover, it has been suggested that the disease is triggered by a massive, unregulated immune response which results in the secretion of a group of inflammatory cytokines (TNF-alpha, IL-1 and IL-6) which produce a clinical emaciation of animals and therefore cachexia.. Moreover, cachexia is mainly the result of secretion of high levels of “cachexins” which are the proinflammatory cytokines such as TNF-alpha, IL-1 and IL-6. Either directly or indirectly the enhanced immune response also results in proliferation of PCV-2,. PCV2 virus has already been shown to be correlated with PMWS disease. Therefore, the current study was aimed at determining the sequence of immunological (secretion of TNF-α, IL-1 and IL-6) and virological (presence and quantity of PCV-2 DNA in serum) events in a natural outbreak of PMWS, and to compare the PCV-2 isolates obtained from clinically affected as well as normal pigs from the same outbreak by whole- genome sequencing.

Objectives:

1. To determine the sequence of immunological (secretion of TNF-α, and IL-1) and virological (presence and quantity of PCV-2 DNA in serum) events in a natural outbreak of PMWS during an ongoing PRRSV transmission and persistence study.

2. To compare the PCV-2 isolates obtained from clinically affected as well as normal pigs from the same outbreak by whole- genome sequencing.

Materials & Methods:

Animals, housing and related research study: The experimental study design of the PRRSV transmission and persistence study where a PWMS outbreak occurred was published (Cano et al., 2007). To summarize the related research project included a total of 360–7 to 8 week old pigs which were inoculated and allocated in six different groups (Treatment groups A-F); following a protocol of vaccination and challenge with virulent PRRSV

strains (Table 1). Groups of 10 PRRSV-naïve sentinel pigs were brought 30 days after the last vaccination into each treatment group. Sera samples were collected at 0, 3, 7, 14, 37, 67, 97, and 127 days post inoculation (DPI), and at 0, 3, 14, and 30 days after introduction from the treatment group and the naïve pigs, respectively. All sentinel pigs were removed, slaughtered and sampled at 30 days after introduction to the treatment group. The end time point for the study was at 127 days post PRRSV inoculation.

Table 1. Experimental design of the PRRSV research study

Group	DPI				
	0	7	37- 67	67- 97	97 - 127
A	MN-30100 inoculation		S1	S2	S3
B	MN-30100 inoculation	MLV	S1	S2	S3
C	MN-30100 inoculation	MLV	MLV	S1	S2
D	MN-30100 inoculation	MLV	MLV	MLV	S1
E	Sham inoculation		S1	S2	S3
F		MLV	S1	S2	S3

Note: DPI: Days post-inoculation; MLV: time of PRRS MLV application; S1, S2, or S3: Groups of 10 sentinel pigs that were placed in contact with originally inoculated pigs (MN-30100) for 30 days and then removed, slaughtered and sampled. Group A: wild type virus infection only (positive control) (n=80); Group B: wild-type virus plus one dose of MLV (n=80); Group C: wild type virus plus two doses of MLV (n=80); Group D: wild-type virus plus three doses of MLV (n=80); Group E: negative control (n=12); Group F: MLV vaccine only (n=20).

Description of the outbreak:

The PMWS outbreak was observed on a group of pigs that were part of a PRRSV transmission and persistence study on the University of Minnesota Swine Disease Eradication Farm. Within a four months period this group of pigs experienced a high mortality (21%). and (34%) of the pigs were clinically affected with severe wasting and a significantly reduction in growth. Based on diagnostic submission of animal the diagnosis of the “outbreak” was determined to be PCV2 induced PMWS.

PCV-2 diagnostic testing.

In order to determine the sequence of virological events, a previous published protocol for PCV-2 detection (Opriessnig, et al., 2003) was adapted and followed with minor modifications. Furthermore, the performance of

this technique was validated with a plasmid-cloned PCV2 genome, two different PCV-2 isolates grown in cell culture and field samples.

Samples used for PCV-2 detection*:*

A total of two hundred and twelve sera samples collected from two sentinel group (S1 and S2) of pigs at 0, 3, 14 and 30 days after introduction to the treatment group (A, B, E) were tested for PCV2. Also, a total of three hundred and twenty sera samples collected from four treatment groups of pigs (A, B, C, D) at 0, 3, 7, 14, 37, 67, 97, and 127 days post inoculation (DPI) with PRRSV MN-30100 were also tested for PCV-2 .

Immunological events:

Cytokine testing and sera samples used:

In order to determine the sequence of immunological events, two cytokines (TNF-alpha and IL-1) were measured following the procedures of two commercially available kits (porcine TNF-α/TNFSF1A catalog number: DY690B and porcine IL-1β/ IL-1F2 catalog number: DY681). We were not able to test for IL-6 due to the absence of an available reagents for setting up an ELISA for porcine IL-6.

Samples used for cytokine detection*:*

Samples obtained from two selected sentinel groups (S1 and S2) from treatment group A, B, and E were evaluated for inflammatory cytokine concentrations. The selected sentinel groups were introduced in treatment groups at 37 (S1) and 67 (S2) PRRSV post inoculation days and were sampled at 0, 3, 14, and 30 days after introduction to the treatment group. A total of 147 samples were tested for cytokine , specifically TNF-alpha and IL-1. (see section VII for results in immunological events)

Progress achieved toward meeting the objectives:

Both of the outline objective 1 was totally completed However we encountered a number of problems in undertaking the outlined studies.. We are describing in this report some of the problems encountered.

1. Some inconveniences were experienced during the validation process of cytokines detection (TNF-alpha, IL-1 and IL-6); including:

a. The TNF-alpha kit (R&D systems) used at first in the validation had a sensitivity of above 250pg/mL and therefore it limited the detection of positive results of TNF-alpha lower than 250pg/mL in the samples. A total of 96 samples were tested with this kit (data not reported due to the lack of sensitivity and quality control issues). For example, the positive control of the kit was not strong enough for its supplied concentration, and this was evidenced later when a positive in house control was produced in our laboratory by stimulating alveolar macrophages with LPS.

b. Limited available kits were offered in the market, so the process of validation of the only two available kits for TNF-alpha detection specific for swine serum in our laboratory was time consuming for the protocol standardization (January 2006 to July 2007).

c. After obtaining a new kit for TNF-alpha with a higher sensitivity (Endogen Kit), some samples were not available due to the limited serum collected from the animals or the samples were already used for other testing, including: PRRSV antibody detection, PCV-2 genome copy detection, TNF-alpha and IL-1 detection.

d. The more sensitive kit used for TNF-alpha detection had a high coefficient of variation between plates (25%) and within plates (27%). The percentage of recovery calculated after spiking three samples with known concentrations of TNF-alpha was 21%.

e. Moreover, during the process of IL-1 detection we faced that: the standard curve of the kit had quality control issues, For example, the OD value (0.86) levels obtained for the highest concentration (4000 pg/mL) of the standard curve were almost two times lowered than the reported in the protocol. The IL-1 values obtained from the samples were mostly negative with a 0D mean of all samples tested ≤ 0.02. Also, the lowest OD value for the least concentrated standard curve (62.5 pg/mL) was 0.035; leading to a tight range between standard values for further concentration calculation with the point to point logistic model constructed.

f. Most of the samples were tested twice due to the low sensitivity of the first test for TNF-alpha and the negative results obtained from IL-1 detection. However, the authors were able to include in this report the results of a total of 147 and 81 samples for TNF-alpha and IL-1 quantification, respectively (see section VII for results in immunological events).

PCV-2 sequencing:

Whole - genome sequencing was performed on selected isolates from the outbreak. The DNA sequence of isolates from the natural outbreak was not found to differ from other isolates on record.

Statistical analysis:

Mean and standard errors were used to describe the data obtained for TNF-alpha levels and PCV-2 load from serum samples. The number of PCV-2 genome copies/mL was log-transformed to normalize the data prior any analysis. Log PCV-2 genome copies/mL and TNF-alpha were compared among groups and among days post introduction in inoculated (vaccinated and no vaccinated) and no inoculated groups with PRRSV by one-way ANOVA. A Pearson correlation coefficient was computed to assess the relationship between TNF-alpha and PCV-2 at day 0, 3, 14 and 30 among the sentinel group of pigs introduced to the treatment groups A and B at 67 days post infection. All analyses were performed using SAS software (Statistical Analysis System, version 9.1; SAS Inst. Inc. Cary, North Carolina).

Results:

Virological events:

Mean and standard errors of log PCV-2 are presented in Table 2 and illustrated in Figure 1. The proportion of infected pigs with log PCV-2 per sentinel group and treatment group every sampling day are summarized (Table 2).

S1: group of sentinels introduced to A, B and E treatment at 37DPI.

No statistically significant differences in mean log PCV-2 were observed among sentinels groups introduced to treatment groups (A and B) and to control group (Group E) at 0 dpi ($P \geq 0.05$) The mean in log PCV-2 decreased (group A) and increased (group B) among sentinels viremic pigs from groups introduced to treatment groups at 3dpi respectively ($P \geq 0.05$) , remaining with no significance difference throughout 14 and 30dpi ($P \geq 0.05$). Control sentinel pigs (group E) were not statistically significant from the viremic sentinel group pigs A and B. The proportion of viremic (PCV-2) pigs among group S1 remained with no significant difference over time to 14dpi ($P \geq 0.05$). At 30dpi the proportion of viremic (PCV-2) pigs was decreased when compared to the proportion of viremic (PCV-2) pigs at 14dpi ($P \leq 0.05$). Moreover, at 0dpi 78% of all the sentinel pigs where

PCV-2 positive with mean log PCV-2 values of 4.65; (min: 3.62; max: 6.09). At 30dpi 27% of the sentinel pigs where PCV-2 positive with mean log PCV-2 values of 4.61 (min: 3.75; max: 5.75).

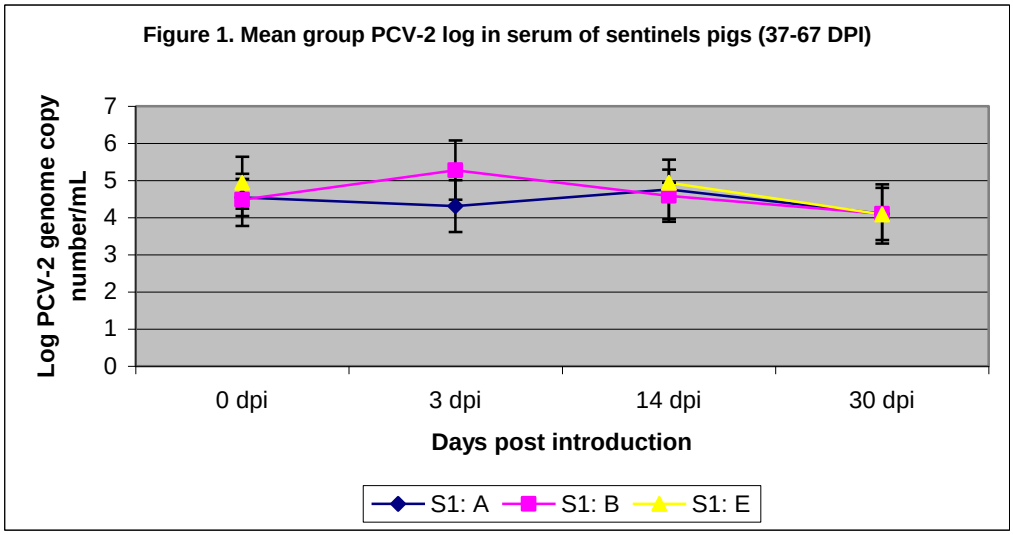
S2: group of sentinels introduced to A, B and E treatment at 67DPI.

No statistically significant differences in mean log PCV-2 was observed among sentinels groups introduced to treatment groups (A and B) ($P \geq 0.05$). Negative control pigs (group E) remained PCV-2 negative throughout the study. The mean in log PCV-2 remained with no statistical difference among sentinels groups introduced to treatment (A and B) throughout the 30 days of exposure ($P \geq 0.05$). The proportion of viremic (PCV-2) pigs among group S2 remained with no significant difference among over time to 30 dpi ($P \geq 0.05$). At 0dpi 30% of the sentinel pigs were PCV-2 positive with mean log PCV-2 values of 4.9 (min: 4.1; max: 5.99). At 30dpi 30% of the sentinel pigs were PCV-2 positive with mean log PCV-2 values of 4.61(min: 3.75; max:3.75).

Table2. PCV Virological response of sentinel pigs introduced into PRRVS inoculated and non-inoculated groups.

	dpi							
	0		3		14		30	
Group	P	VL	P	VL	P	VL	P	VL
S:1 - A	9 /10	4.54 ± 0.5	9/10	4.31 ± 0.7	7/10	4.76 ± 0.8	2/9	4.1 ± 0.7
S:1 - B	7/10	4.48 ± 0.7	7/10	5.28 ± 0.8	7/10	4.59 ± 0.7	2/9	4.1 ± 0.8
S:1 - E	9/12	4.94 ± 0.7	na	na	0/12	0 ± 0	0/12	0 ± 0
S:2 - A	4/10	4.5 ± 0.7	3/10	4.31 ± 0.7	4/10	4.76 ± 0.7	4/10	4.45 ± 0.7
S:2 - B	4/10	5.31 ± 0.9	3/10	5.14 ± 0.8	3/10	4.86 ± 0.8	4/10	4.78 ± 0.8
S:2 - E	0/6	0 ± 0	na	na	0/6	0 ± 0	0/6	0 ± 0

Note: **P:** number of infected pigs/ number of tested pigs; **VL:** viral load (log10 copy/mL/group) mean ± SE; groups with **S1:** introduced 37 days post inoculation; **S2:** introduced 67 days post inoculation; **A:** sentinels commingled with pigs infected with a PRRSV field strain (MN-30100);**B:** sentinels commingled with pigs infected with a PRRSV field strain (MN-30100) and vaccinated with a modified-live PRRSV vaccine; **E:** sentinels commingled wit PRRSV-free pigs.

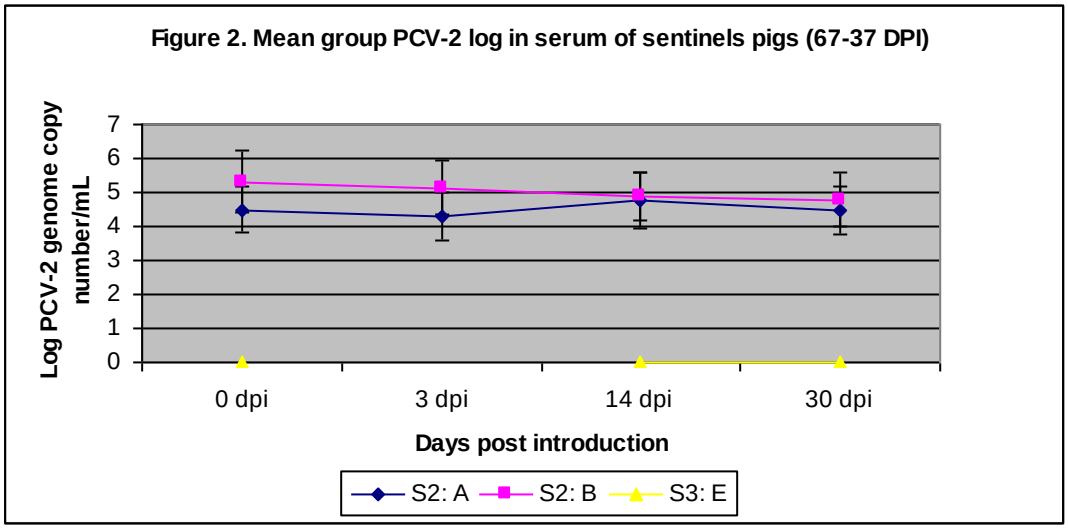


Note: Values of Log PCV-2 genome copy are group means ± SE.

Group A: sentinels commingled with pigs infected with a PRRSV field strain (MN-30100).

Group B: sentinels commingled with pigs infected with a PRRSV field strain (MN-30100) and vaccinated with a modified-live PRRSV vaccine.

Group E: sentinels commingled wit PRRSV-free pigs.



Note: Values of Log PCV-2 genome copy are group means ± SE.

Group A: sentinels commingled with pigs infected with a PRRSV field strain (MN-30100).

Group B: sentinels commingled with pigs infected with a PRRSV field strain (MN-30100) and vaccinated with a modified-live PRRSV vaccine.

Group E: sentinels commingled wit PRRSV-free pigs.

Immunological events:

Selected samples from entire collection of sequential time points were examined for IL-1 and TNF-alpha. In the first instance, eight samples were positive for IL-1; with a mean of 98.4 pg/mL, the highest value detected was142.69 pg/mL, out of the 81 samples. Due to high percentage of negative sample (90.1%), we decided to not perform further testing for IL-1.

Moreover, Mean and standard errors of TNF-alpha levels are presented in Table 3 and illustrated in Figures 3 (S1 group) and 4 (S2 group).

Table 3. TNF-alpha levels in pigs introduced into PRRSV inoculated and non- inoculated groups

TNF-alpha (pg/ml				
Group	0d	3	14	30
S:1 - A	587.6 ± 48.25	217.2 ± 10.0	423.5 ± 39.5	332.6 ± 18.4
S:1 - B	516.4 ± 48.25	281.0 ± 46.4	340.3 ± 27.9	329.9 ± 51.3
S:1 - E	na	na	204.3 ± 9.7	287.7 ± 35.2
S:2 - A	124.2 ± 10.7	217.2 ± 10.0	423.5 ± 39.5	332.6 ± 18.4
S:2 - B	218.2 ± 28.2	281.0 ± 46.4	340.3 ± 27.9	329.9 ± 51.3
S:2 - E	223.2 ± 15.6	na	204.3 ± 9.7	287.7 ± 35.2

Note: mean ± SE; groups with **S1:** introduced 37 days post inoculation; **S2:** introduced 67 days post inoculation; **A:** sentinels commingled with pigs infected with a PRRSV field strain (MN-30100);**B:** sentinels commingled with pigs infected with a PRRSV field strain (MN-30100) and vaccinated with a modified-live PRRSV vaccine; **E:** sentinels commingled wit PRRSV-free pigs.

The overall mean of TNF-alpha concentration among all samples evaluated under the conditions of this study was 284 pg/mL. Among the samples tested for TNF-alpha; only one animal had values of TNF-alpha lower than 15.75 pg/mL. Moreover, this animal was tested negative for PCV-2. However, there was no correlation between TNF-alpha levels and PCV-2 log copy number at 0, 3, 14, and 30 days post introduction for naïve piglets introduced in the treatment groups (A and B) at 37 days post inoculation (P value >0.05). TNF-alpha levels among the sentinel groups (S1 and S2) are graphically illustrated in Figures 3 and 4.

Figure 3. Mean group TNF-alpha levels in serum of S1 (37-67DPI) sentinel pigs

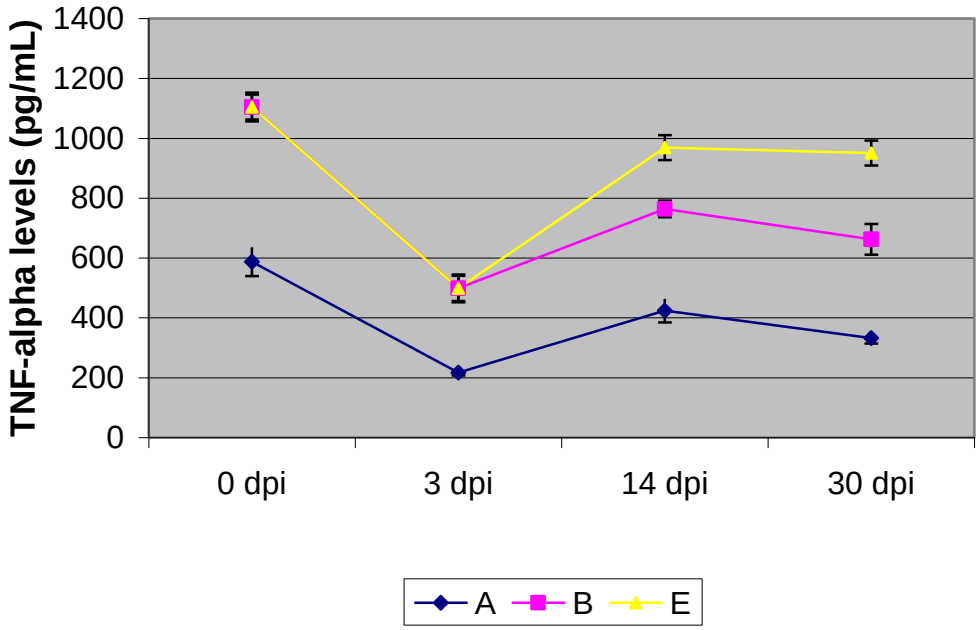
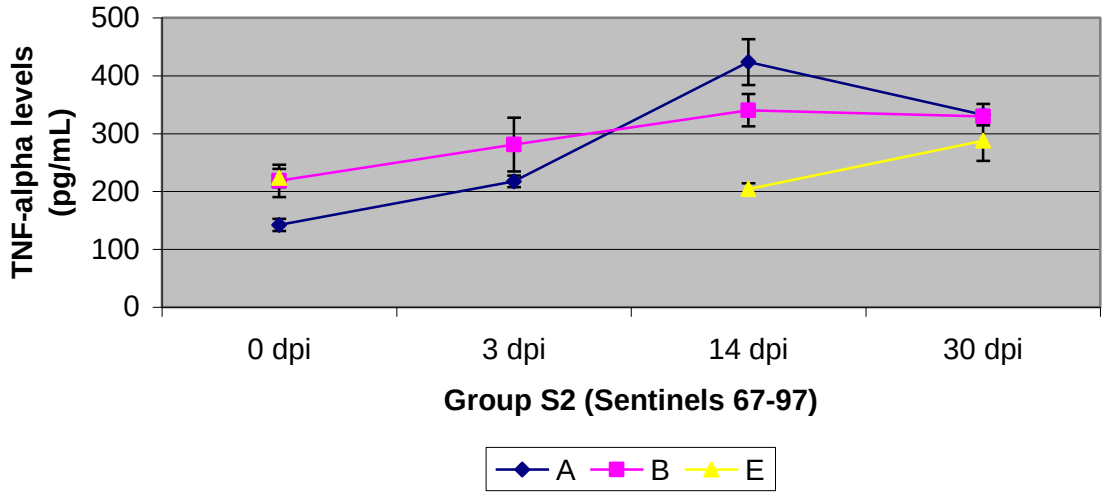


Figure 4. Mean group TNF-alpha levels in serum of S2 (67 to 97 DPI) sentinel pigs



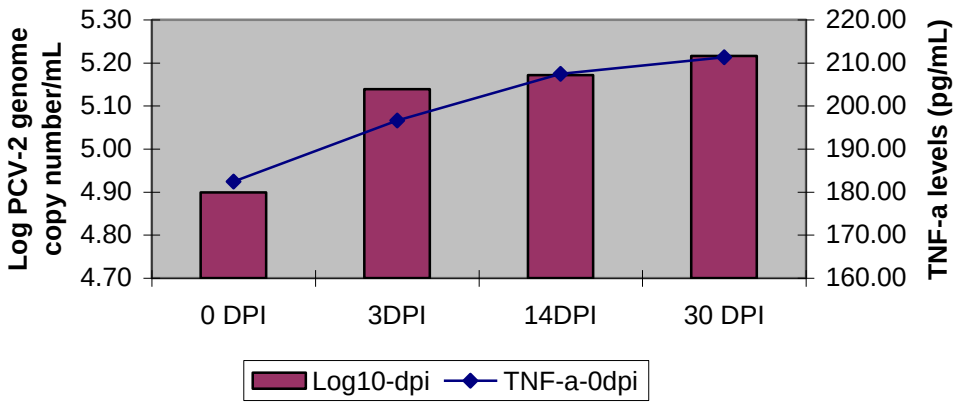
Group A: sentinels commingled with pigs infected with a PRRSV field strain (MN 30-100).. **Group B:** sentinels commingled with pigs infected with a PRRSV field strain (MN30-100) and vaccinated with a modified-live PRRSV vaccine. **Group E:** sentinels commingled wit PRRSV-free pigs.

Relationship between TNF-alpha and PCV-2 viral load among viremic pigs:

The relationship of virus load and TNF concentration was determined in PCV viremic pigs.. A narrow correlation was found in pigs all (viremic and no viremic pigs) over time r= , Fig 5.), For a better descriptive analysis we prepared a small data set that included PCV-2 viremic pigs; animals with any missing value on 0, 3, 14 and 31 dpi were excluded of the data set. Data regarding PCV-2 viral load and TNF-alpha level among all day of testing was available for six pigs. The mean PCV-2 load and the TNF-alpha levels detected in PCV-2 viremic pigs among the 30 days period after introduction to the treatment groups is hown in Fig 5., the log PCV-2 genome copy among time on one Y axis and the TNF-alpha level on the other Y axis. Moreover, there was an association between levels of TNF-alpha and PCV-2 load among PCV-2 viremic pigs at 0dpi (coefficient correlation: 0.67, P≤0.05); 3dpi (coefficient correlation: 0.83, P≤0.05). At 14dpi and 30dpi coefficient correlation are reported as 0.37 and 0.49 respectively.

However further research addressing this outcome is needed for a better understanding in the immunological response due to PCV-2 infection.

Figure 5. Immunological and virological sequene TNF- and PCV-2 - group S2: A and S2: B



Discussion:

This study aimed to describe the framework for immunological events that lead to the development of PMWS clinical signs in pigs naturally infected with PCV-2. TNF-alpha levels were consistently detectable in all our samples except for one, which was also being to PRRSV and PCV-2. The E group of our study was negative

to PCV-2 and PRRSV but showed the highest levels of TNF-alpha. The increased values among the control group could be perceived as frustrating except when you consider the process of secretion of TNF-alpha. The proinflammatory cytokines are liberated in situations of stress and these animals were recently moved and mixed with older animals; which might be a strong argument for the lack of difference among the groups. Also, limited sample size among each category group limited our study to perform a logistic regression analysis for controlling to other variables, such as the co-infection with PRRSV and vaccination status. After analyzing the data and looking only to viremic pigs we were are able to see that TNF-alpha secretion increased as PCV-2 load increased. Due to being a natural infection of PCV2, in this study the time point of the infection with PCV-2 was unknown and the description of the TNF-alpha levels in this study are only driven throughout the time that animals were initially introduced to an ongoing experiment.

Selected References:

Cano, J.P. et al., 2007. Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* (25):4382-4391.

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