RESEARCH REPORT
SWINE HEALTH

Title: Identification of a putative viral co-factor different from PCV2, in animals with PMWS.
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Industry Summary:

During the last two years, the availability of effective vaccines against PCV2 and their extensive successful use under field conditions to control PMWS have allowed to convincingly demonstrate that porcine Circovirus fulfills an important etiological role in this multi-factorial syndrome. In spite of this established importance of PVC2 for PMWS, the disease continues to be considered multi-factorial and the possibility of other infectious co-factors being involved in PMWS remains a lively possibility. In this research we took advantage of a very well characterized set of samples from a PMWS-affected herd. Using highly sophisticated molecular biology techniques we attempted to ascertain if an unknown new viral agent would be involved in these well recognized cases of PCV2-associated PMWS. Our results provide evidence that important molecular disorders typical of chronic inflammations and other pathologic (not necessarily infectious) processes may also be involved in PMWS. However, we have been unable to demonstrate the presence of any other novel, yet unknown infectious agent involved din the etiology of PMWS.

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Scientific Abstract:
Despite the lack of correlation between PCV-2 infection and PMWS, PCV-2 is recognized as an important agent. In pursuit of identifying novel viral co-factor we analyzed well characterized PMWS associated tissue samples which may be responsible for disease progression. To this end, different tissues samples (Lymph node, tonsil, liver, lungs etc) from affected animals derived from three independent farms were collected for our study. The results obtained from Subtractive PCR hybridization indicate presence of activated host mitochondrial genes. Also in few cases the bacterial ribosome sequences have been noticed. The activation of mitochondrial genes indicate that the animals associated with PMWS undergo severe stress. However the complete genome sequencing analysis data indicated possible link between mutations within PCV2 capsid region and disease outcome, therefore supporting the hypothesis of strain variation that may constitute the factor that trigger switch from PCV2 infection to PMWS disease. Furthermore the virus isolation attempts were carried out using various cell lines. Although the cell lines used in our study represent susceptibility to wide range of known viruses failed to provide any evidence of a novel viral co-factor.

Introduction: An overview of the researchable question and its importance to producers.

Postweaning multisystemic wasting syndrome (PMWS) was identified several yrs ago which is often associated with PCV2 infection. It appears infection with PCV2 is crucial for development of PMWS but this virus alone may not be the only causative factor that triggers PMWS. Various studies have provided some assessment of the risk factors involved in the development of PMWS but specific co-factors involved in generating the disease remains to be characterized.

Several studies indicate infections with other viruses i.e. porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV) could aggravate the disease outcome. In the order to test a novel viral co-factor associated with PMWS, we performed several studies from tissue samples from well defined farms that are associated with PMWS. These objectives involve subtractive PCR hybridization, phage display library and Virus Discovery cDNA Amplification (VIDISCA) from the above described tissue samples.

The role of specific genotypes of PCV2 has been implicated in many of the earlier studies and currently debated. Although majority of these studies indicate no association of PCV2 genotype and disease progression but a recent studies from Timmusk et al revealed nucleotide and amino acids differences in the samples collected from PMWS positive and negative animals. Keeping this information in mind we cloned and sequenced the entire genome of PCV2 from animals originated from two farms to analyze the possible effect of PCV2 genotype and disease association.

Objectives:

The following objectives were proposed for this study and performed:

A. Subtractive PCR hybridization

B. Phage display library
C. Virus Discovery cDNA Amplification (VIDISCA)

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

A. Subtractive PCR hybridization

A.1. Tissue samples

Swine organ tissues consisted of ileum, tonsil, lung, spleen, lymph nodes, kidney and intestines. These samples were collected from each individual pig which expressed symptoms of PMWS. Total of 18 pigs were tested from three pig farms A, B and C (six animals from each farm) were selected which had the history of affirmatively positive PMWS (kindley provided by Dr. Montse Torremorrell). Tissue samples were submerged in RNAlater Tissue Solution (Ambion, Cat # AM7020) during transportation from farms to the laboratory, and then they were store at -80^{o}C until being tested.

Before doing Representational difference analysis (RDA) method, all obtained samples had been tested by PCR method to confirm positive with PCV and PCV2; but simultaneously negative to PRRSV, *Mycoplasma hyopeumonia* and SIV. Moreover, the samples were also determined free or non – significant bacterial contamination by inoculating in bacteria growth medium. The qualified tissue samples then were used as Tester in RDA.

The Driver tissue samples were collected from a healthy pig that showed negative results for PCV2 and PRRSV. The healthy tissue samples were kindly provided by Dr. Richard A. Hesse, Kansas State University, KS.

A.2 RNA preparation

In order to purify RNA, all tissue organ of individual pig were spooled and used about 200mg. Total RNA isolation was performed using TRIzol reagent (Invitrogen, Cat.No 15596-026) according to the manufacturer's instruction. Samples from farm A, B and C were designed A, B and C.

A.3 Representational difference analysis method (RDA)

The entire procedures of RDA was performed as per PCR-Select cDNA Subtraction Kit (Clonetech) protocol. In brief following steps were used:

- cDNA synthesis: RNA of Driver and Tester was used to synthesize first strand of cDNA using AMV Reverse Transcriptase (20units/reaction) at 42^{o}C in 90 minutes. The second strand of cDNA was implemented by an enzyme cocktail consisting of DNA polymerase I (24 units/reaction); RNase H (1units/reaction) and *E. coli* DNA ligase (4.8 units/reaction) at 16^{o}C in 2 hours. The synthesis was completed by T4 DNA polymerase (6 units/reaction) at 16^{o}C in 30 minutes.

- *Rsa*I digestion: double-stranded cDNA of Driver and Tester then was digested by RsaI restriction enzyme (15units/reaction) at 37^{o}C during 1 hour and 30 minutes.

- Adapter ligation: after digestion, the Tester cDNA was partially ligated to either of two discrete adaptors 1 and 2R at 16^{o}C overnight. These two adaptors contained T7 promoter and complementary sequences for the primers using in PCR reactions.

- Two rounds of hybridization: each adaptor – ligated Tester subsequently was hybridized two times to the appropriate Diver in order to eliminate all similar fragments and seize the differential expressed sequences between the two clusters of cDNAs.

- Two rounds of PCR: the hybridized cDNA then was amplified by the first PCR reaction using 50X Advantage cDNA Polymerase Mix (Clontech, Cat#639105) with thermal cycling of 94^{o}C/27 seconds; 94^{o}C/10 seconds; 66^{o}C/30 seconds; 72^{o}C/1.5 minutes for 27 cycles. Products of the first PCR were then magnified in the second nested PCR with the same components of reaction and thermal cycling except for the nested primers that replaced the primers in the first PCR.

A.4 Cloning, sequencing and sequence analysis

The differentially expressed cDNAs were gel purified using glass slurry method and was eluted in 15µl distilled nuclease free water. Each cDNA fragment was ligated into pGEM-T Vector System (Promega, Cat#A3600). Clones were screened and selected on LB agar plates containing ampicillin. The sequence of each plasmid DNA containing individual cDNA fragment was determined by T7 or SP6 promoter primers. To sequence the full length PCV2 genome, we used the above mentioned primers in combination with PCV2 specific internal primers that were commercially synthesized.

B. **Phage Display Library**

Phage display describes a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage, resulting in display of the fused protein on the surface of the virion, while the DNA encoding the fusion resides within the virion. In our protocol we used Ph.D.-C7C™ Phage Display Peptide Library Kit which is based on a combinatorial library of random peptide 7-mers fused to a minor coat protein (pIII) of M13 phage. A brief protocol is mentioned below:

1. Total RNA was isolated from the above group of animals as above and cDNA was synthesized. This cDNA was mixed with three times excess of extension primer in total volume of 50 µl. Before extension the reaction mixture was heated at 95C for 5 mins and slowly cooled down to RT. The extension reaction was performed as follows.

H_20 119 µl
10X Klenow buffer (NEB) 20 µl
Annealing reaction 50 µl

10 mM dNTP's 8 µl
Klenow fragment (10 U/µl) 3 µl
Total reaction volume 200 µl

The reaction was incubated at 37C for 30 min and inactivated at 65C for 15 ins. Following this the duplex DNA was ethanol precipitated used for digestion with KpnI and EagI restriction enzymes as follows

Extension reaction 196 µl
H_2O 159 µl
10X NEBuffer 1 40 µl
KpnI (10 U/µl) 5 µl
Total volume 400 µl

After 3hrs of incubation at 37C the buffer conc was adjusted to NEBuffer 3 and 5 ul of EagI enzyme was added and the reaction was further incubated for 3 hrs at 37C. The DNA was purified by phenol/chloroform extraction, chloroform extraction and ethanol precipitation.

2. The digested DNA was resolved by running 8% nondenaturing polyacrylamide gel along with a molecular weight markers. The DNA was stained with ethidium bromide and excised from the gel. The DNA was eluted in 100 mM NaOAc, pH 4.5, 1 mM EDTA, 0.1% SDS at 37°C.

Again the eluted DNA was purified phenol/chloroform extraction, chloroform extraction and ethanol precipitation and resuspended in 50 µl of TE

This DNA was ligated using supplied vector (vector to insert ratio 1:5) in a 20ul reaction volume using 200 units of T4 DNA ligase (NEB) and incubated overnight at 16°C.

3. The ligation mix was heat inactivated at 65°C for 15 minutes, then electroporate 1.0 µl of each into electrocompetent ER2738 (NEB #E4104S). Count the plaques and Recover phage from supernatant by adding 1/6 volume of PEG/NaCl and incubating overnight at 4°C. Pellet phage by centrifugation for 15 minutes at 10,000 rpm. Resuspend in 100 ml TBS, spin out residual cells, and reprecipitate phage from supernatant with PEG/NaCl. Resuspend final library in 10–40 ml TBS by gentle rocking at 4°C for 24–48 hours and titer again.

Panning the Phage:

The pool of tissue samples were homogenized in PBS and cleared of cell debris by centrifuging at 10,000 g for 10 min. The supernatant was collected and coated on ELISA plate in a 100 mM bicarbonate buffer overnight at 4C in humidified chamber (150ul each well). Pour off the coating solution and add 200 ul each well with Blocking Buffer (0.1 M NaHCO3 (pH 8.6), 5 mg/ml BSA, 0.02% NaN3. Filter sterilize) and incubate at least 1 hour at 4°C.

Discard the blocking solution and wash each plate six times with TBST (TBS + 0.1% [v/v] Tween-20). Dilute 2 x 10^{11} phage with 1 ml of TBST and add 100 µl to each well and rock gently for 60 minutes at room

temperature. Discard nonbinding phage by pouring off and wash plates 10 times with TBST as above. Elute bound phage with 100 µl of elution buffer for 60 minutes and collect supernatant into a microcentrifuge tube.

Titer a small amount (~1 µl) of the eluate and rest amplify using ER2738 E. coli. Precipitate phage by adding 1/6 volume of PEG/NaCl at 4°C overnight. Spin PEG precipitation 15 minutes at 10,000 rpm, at 4°C. Decant supernatant, re-spin briefly, and remove residual supernatant with a pipette and resuspend the pellet in 200 µl TBS, 0.02% NaN3. Titer the amplified eluate as described in General M13 Methods on LB/IPTG/Xgal plates. Store at 4°C.

Again coated a plate for the second and third round of panning with increased concentration of 0.5% Tween20 (v/v) with every additional round to increase the specific binding of phage to the target.

C. **Virus Discovery cDNA Amplification (VIDISCA)**

The above mentioned tissue samples were homogenized in a plastic bag in presence of PBS pH 7.2. The homogenates were collected and clarified to get rid of cell debris. The supernatants were collected and applied on to different cell lines (BhK-21, MDBK, Huh-7, HeLa, 293-T and MARC145 cells) and incubated in CO_2 incubator for 3-4 days. These cells were either examined for appearance of cytopathic effect or freeze-thawed 3 times and supernatant was further inoculated onto fresh cells to observe any cytopathic effect after 4-5 days post inoculation.

Results: Report your research results by objective.

A. Subtractive PCR Hybridization

After two rounds of PCR reactions, 4 differential DNA bands (1, 2, 3 and 4) appeared for each farm A and B, and none for farm C indicating that farm C does not have any distinct expressed DNA (data not shown).

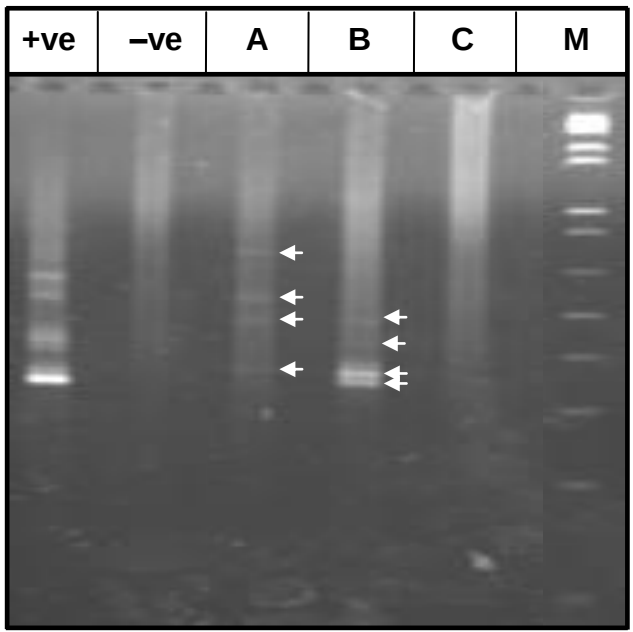

Figure 1. Representative of an agarose gel electrophoresis of differentially expressed cDNA fragments from

As expected there was no signal in negative control while an expected size of differential amplification was observed in positive control. This result clearly indicates the system worked well in our hands and what ever signal we observed in farm A and B are authentic. There was no signal observed in samples tested for farm C.

farms A, B and C. +ve and –ve controls supplied with the kit, M= 1kb plus DNA ladder (Invitrogen). The arrow marked differentially expressed DNA fragments were excised from the gel, purified and cloned for sequencing.

Table 1. Comparison and analysis cloned sequences acquired by RDA from positive PMWS pigs

Clone	Size (bp)	Genbank accession	Database identity	Identification
A1	550	DQ972936.3	99%	*Sus scrofa* breed Type II Lanyu mitochondrion, complete genome
A2	700	AK230698.1	98%	*Sus scrofa* mRNA, clone:AMP010027C02, expressed in alveolar macrophage
A3	800	AF304202.1	100%	*Sus scrofa* breed Landrace mitochondrion, partial genome
A4	850	CT009569.17	94%	Pig DNA sequence from clone CH242-213C10 on chromosome 17, complete sequence
B1	350	AK240465.1	95%	*Sus scrofa* mRNA, clone:UTR010076C08, expressed in uterus
B2	420	AK240465.1	99%	*Sus scrofa* mRNA, clone:UTR010076C08, expressed in uterus
B3	600	AJ551142.1	100%	*Pseudomonas sp*. An1 partial 16S rRNA gene, isolate An1
B4	800	AB275587.1	100%	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: Yasu146

The analysis in the table 1 showed that the majority of sequences including A1, A2, A3, A4, B1 and B2, belongs to swine *Sus scrofa* DNA. They were identified as mitochondrial and chromosomal DNA or mRNA as well. This analytical result was very much similar to what was reported before by Bratanich et al.

However, the two last cDNA clones B3 and B4 are similar to bacterial genes in which, only *Pseudomonas* could be a pathogen in swine. Nevertheless, this finding may be a good contribution to the scenario of multiple pathogenic bacterial co-infection in PMWS observed by other group of researchers.

Complete sequencing of PCV-2 genome from two farms

In order to further investigate the sequence variation in the PCV2 genome in the animals with PMWS, we cloned and sequenced the entire PCV2 genome derived from the above two farms and compared with the reported PCV2 sequence (acc no. NC_005148). We observed 99% homology at the nucleotide level and 100% homology at the amino acid level among the viruses that we cloned from farms A and B. However these viruses have only ~91% homology at the amino acid level when compared with wt sequence (NC_005148) within the capsid region. The results indicate there were only four amino acids changes in the viral replicase region when

compared with the wt seq (NC_005148). Furthermore these changes are classified as conserve changes and may not affect the amino acid properties and hence the overall the basic function of the entire protein remains same. In addition there were number of changes observed within the capsid region of our virus when compared wt seq (NC_005148) as shown below. These changes include all; semi-conserve, conserve and complete change. Some of these drastic changes may affect the protein function. It is easy to speculate that virus with so many mutations in capsid region could have different pathology since capsid protein is involved in virion assembly, release and attachment. However that remains to be investigated. The following result summarizes the amino acid changes that we observed in our studies.

Mutation in the viral replicase region

```
aa pos:    34  77  105  139
NC_005148: E   F   M    H
Farm A:    D   L   I    Y
Farm B:    D   L   I    Y
           :   :   :    :
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Mutation in the viral capsid region

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aa pos:    47 57 59 63 72 76 77 80 86 88 89 91 123 131 136 151 185 190 191 232
NC_005148: A  V  A  S  L  L  D  V  T  K  I  I  I   I   Q   P   M   S   R   K
Farm A:    T  I  R  R  M  I  N  L  S  P  R  V  V   T   L   T   L   A   G   N
Farm B:    T  I  R  R  M  I  N  L  S  P  R  V  V   T   L   T   L   A   G   N
           :  :  -  -  :  :  :  :  :  -  -  :  :   -   -   .   :   :   -   :
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Code description:

"-" means that the residues or nucleotides in that column are not identical.

":" means that conserved substitutions have been observed.

"." means that semi-conserved substitutions are observed.

B. Phage display library

After third round of panning there was no phage bound to the plate hence no further phage amplification was performed. It appears that the number of phage we detected following first and second round were not specific and after increasing the tween20 in wash buffer all the non specific phage were washed out.

C. Virus Discovery cDNA Amplification (VIDISCA)

This objective was based on the assumption that novel co-factor will be further amplified when passed on to cells. To avoid further amplification of PCV2 from these samples we decided not to use the swine cells. In this study we used established cell lines from Bovine origin, MDBK, a cell line from human origin and a cell line that is permissive to PRRSV, a swine pathogen to make sure that we did not miss any novel cofactor. The earlier results after first pass indicated possible development of CPE in HeLa cells. However this result was

sporadic in nature and when passaged further, no CPE was observed even observed for longer periods. The subsequent procedure Virus Discovery cDNA Amplification (VIDISCA) could not be performed since there was no further amplification of any viral cofactor using any of the cell types used. The table below summarizes the results obtained by using different cell lines.

CELL LINE	ORIGIN	RESULT
BHK-21	Syrian baby hamster kidney cells	No CPE
MDBK	Madin-Darby bovine kidney epithelial cells	No CPE
Huh-7	human hepatoma cells	No CPE
HeLa	Human cervix carcinoma cells	No CPE
293-T	Human embryonic kidney cells	No CPE
MARC-145	monkey kidney cells	No CPE

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

This project took a thorough approach to find novel putative cofactor involved with PMWS. However the findings in our laboratory did not indicate any involvement of novel viral cofactor. Although the hyper activation of mitochondrial genes indicate the utter stress conditions of the animals infected with PCV2 but more studies needed to confirm the role of these genes in PMWS.

The complete genome sequence results indicate possible role of certain mutations within capsid region may contribute to disease progression. A similar finding has been reported recently (Lohse et al 2008) that support our results obtained in this study. Further we analyzed the genome variation that may trigger the switch from PCV2 infection to clinical onset. The entire PCV2 genome obtained from organ material was sequenced. To this end we found there are number of amino acid changes in capsid and replicase region when compared with the wt reported sequence. The overall sequence homology at the amino acid level between the two farm animals is 100% but drops to ~ 91% when compared with reported sequence (NC_005148) in the capsid region. The mutation in the replicase region was of conservative in nature while the mutations within the capsid region were more pronounced that may affect protein function.

In conclusion, the present study does not provide any evidence of presence of a novel cofactor in PMWS animals. However the complete genome sequencing data indicate a possible link between genome variation and disease progression in infected animals.

In order to further increase the chances of finding putative cofactor a broad range of cell lines were used for virus isolation assays. The panel of cells used provided a wide variety of target tissues for wide range of virus families. To conclude our findings, we did not find any evidence of the presence of any specific virus that is involved in PMWS. Despite the use of wide variety of cell lines for propagation of putative cofactor, no novel candidate was found.