RESEARCH REPORT
SWINE HEALTH

Title: A modified-live prototype vaccine for PCV-2 in swine - **NPB** #10-047

Investigator: Steven Krakowka, DVM, PhD, Professor

Institution: The Ohio State University

Date submitted: July 1, 2011.

Industry Summary:

1. The overall goal of this project is to test the hypothesis that, "*The critical virulence determinant of swine-virulent PCV2 resides in a linear 3-4 amino acid region in the center of the second immunogenic epitope of PCV2 nucleocapsid protein.*"

2. There are two reasons (academic and practical) that the data generated by this NPB grant are important to producers and the swine industry:

   Firstly, a successful outcome (i.e. archival PCV2 recovered from swine tissues 25-years prior to the first reported cases of postweaning multi-systemic wasting syndrome is avirulent for pigs) provides a credible explanation for the historical presence of avirulent PCV2 in swine and also provides a molecular explanation for the sudden emergence of the porcine circovirus diseases (PCVDs) in global swine populations.

   Secondly, development of an avirulent yet immunogenic and genomically stable PCV2 (i. e. archival PCV2) will provide the industry with a potential candidate modified-live PCV2 for protection of swine against the PCVDs.

3. We have conclusively demonstrated that a PCV2 virus, reconstructed from archival PCV2 DNA sequences recovered from swine in 1970-71 is avirulent for swine, either alone or in gnotobiotic swine infected with this virus and subsequently immune stimulated. This virus is easily propagated in cell culture and is pig-infectious, stable and has a cell target tropism identical to pig-virulent PCV2s.

4. We have also conducted "proof of concept" experiments wherein we have shown that the archival virus does not potentiate PCVD in dually-infected piglets and also can function as a modified-live virus vaccine for prevention of PCVDs in virulent PCV2-challenged piglets.

Key words: gnotobiotic swine, porcine circovirus type 2, PCV2, nucleocapsid protein, postweaning multisystemic wasting syndrome, PMWS.

Scientific Abstract: Porcine circovirus type 2 (PCV2) was present in swine populations as an asymptomatic infection at least 25 years prior to the first reported case of PCV2-associated postweaning multisystemic wasting syndrome (PMWS). Viral sequences were amplified from frozen archived (1970-1) porcine tissues and the complete genome of archival PCV2 was deduced from these fragments. While the ORF1 gene product (viral DNA replicase) was homologous to contemporary PCV2 ORF1 sequences, a consistent linear nine-base sequence difference was found in positions 1331 through 1339 of the ORF2 nucleocapsid gene. The deduced amino acid sequence from these base changes alters the nucleocapsid conformation in the second immunogenic epitope from a hydrophobic (contemporary PCV2) to a hydrophilic (archival PCV2) configuration. Since archival PCV2 was, *de facto*, avirulent, these molecular sequence data suggest that the critical viral molecular component of PCV2 virulence is associated with this novel archival base sequence and subsequent amino acid difference.

To test this hypothesis, cloned engineered archival and cloned contemporary PCV2 genomes were constructed wherein the ORF1 gene (viral DNA replicase) was identical in each clone and the ORF2 gene (nucleocapsid protein) was sequence-identical in both clones except for the nine-base difference in positions 1331-1339, corresponding to archival and contemporary PCV2 viruses respectively. Clones were transfected into porcine kidney (PK) 15 cells and, after sequence confirmation, further passed in PK15s and also 3D4/2 porcine alveolar macrophages. Virulence trials in gnotobiotic piglets were conducted with these cloned PCV2s. The resultant data support the hypothesis that the conversion of avirulent archival PCV2(s) into virulent contemporary PCV2 virus(es) was accompanied by mutational event(s) within ORF2 sometime after 1971 and that this mutation in part, accounts for the sudden emergence of PMWS in global swine populations in 1998-2000.

Introduction: Porcine circovirus 2 (PCV2) is a single-stranded circular DNA virus belonging to the genus *Circovirus*, family *Circoviridae*. First identified in western Canada in 1996, the primary disease presentation of PCV2 infection is post-weaning multisystemic wasting syndrome (PMWS), a clinical syndrome of progressive wasting and increased susceptibility to other infectious diseases (Allan and Ellis, 2000). PMWS is characterized by generalized lymphoid de-pletion, disseminated angiocentric granulomatous inflammation and organ failures of the liver, renal, respiratory and enteric tracts. Experimentally, PCV2 is pathogenic in gnotobiotic piglets that are immune stimulated shortly after infection, immune suppressed with cyclosporine, when co-infected with other swine pathogens or when given certain swine bacterins.

Most experimental studies have focused upon host, environmental and more recently, viral genetic variations as components in the induction of PMWS. Although external variables play important potentiation role(s) in the genesis of the PCV2-associated diseases (PCVDs), intrinsic viral virulence determinants are also pivotal for PCV2 pathogenicity. This concept is broadly supported by the nucleotide sequence and resultant structural differences between avirulent PCV1 and virulent PCV2. The former is a nonpathogenic swine virus whereas the latter is the necessary cause of PMWS. The percent nucleotide sequence homology between PCV1 and PCV2 is high (82%) for ORF1 whereas homology in the nucleocapsid protein ORF2 gene is low, 60% or less. Thus, in this example, nucleocapsid differences are responsible for the virulence properties of types 1 and 2 porcine circoviruses.

Amongst contemporary (con) pig-virulent PCV2 global isolates, the ORF2 (nucleocapsid protein) base sequence is highly (95-100%) conserved. Genomic stability of ORF2 facilitated development of

inactivated vaccine products for the prevention of PCVDs. Yet in spite of this molecular similarity, resurgence of PMWS cases in Canada in 2004-5 was strongly associated with recovery of a “new” PCV2 genotype that differed from the previously characterized PCV2 isolates by a new restriction fragment length polymorphism (RFLP) in the newer isolates (Carmen et al, 2006). By epidemiology, the presence of the “new” PCV2 genotype in PMWS-affected Canadian swine was associated with these PMWS outbreaks, even in herds subclinically infected with classic PCV2s. Subsequent exhaustive PCV2-ORF2 sequence and RFLP studies in Sweden and elsewhere confirm the original RFLP observation. As a result, PCV2 nomenclature was revised in 2008 by separation of the PCV2s into two genogroups or clades referred to as PCV2a and PCV2b. Isolates retaining the original RFLP pattern including most of the original PCV2 strains were assigned to genogroup PCV2a. PCV2b designated the new RFLP-defined PCV2 isolates recovered primarily from diseased swine after 2005. While clinical observations in naturally infected herds suggested that PCV2b viruses are more virulent than are the PCV2a viruses, this belief is not completely supported when the two genogroups are experimentally compared to each other in virulence trials. In fact, the observed enhanced pathogenic potential in herds that experience PCV2b outbreaks can be replicated experimentally in gnotobiotic swine that have subclinical PCV2a infection prior to challenge exposure with PCV2b, suggesting that the PCV2 genogroups likely cooperate with each other in disease potentiation within dually infected swine.

The sudden emergence of PCVD-PMWS is enigmatic, particularly in light of the fact that a high incidence of PCV2-specific serum antibodies are present in archived porcine sera collected twenty-five years *prior* to the first reported PMWS cases. These serologic data are reinforced by demonstration of PCV2 DNAs in archived porcine tissues. Thus, subclinical infection with PCV2 was a common event in swine well before 1998-2001. One explanation for the global emergence of PMWS is that nonpathogenic or archival (arch-) PCV2s experienced critical genomic mutation(s) sometime after 1970-71. Dissemination of potentially swine-virulent or contemporary (con) PCV2s into global swine operations was accomp-lished by exchange of breeding stock, semen or other husbandry activities. Ultimately, subclinical infection with con-PCV2 was expressed as overt PMWS after swine management conditions changed to favor induction of PCVDs. To investigate this possibility, porcine tissues collected in 1970-1 and stored (-70^{o}C) in the Veterinary Sciences Division, Department of Agriculture of Northern Ireland (DANI), now Agri-Food and Biosciences Institute (AFBI), Belfast, Northern Ireland, were tested for the PCV2 DNAs by PCR methods. From the PCR fragments amplified and recovered, an archival sequence for the entire PCV2 genome was reconstructed and this archival (arch) sequence was compared to sequence data compiled on con-PCV2a viruses. Reconstructed arch-PCV2 sequences were homologous to various con-PCV2 DNAs except for a linear nine-base nucleotide sequence difference located near the C-terminus end of ORF2. These nine-bases translated into a contiguous three- amino acid (residues 133-135) change in the primary structure of nucleocapsid protein. When a fourth common lysine residue is included, a hydrophobic sequence in alanine-threonine-alanine-*lysine* typical of contemporary (con) PCV2s, is created
whereas a hydrophilic sequence (threonine-glycine-asparagine-*lysine),* char-acterized the archival (arch) PCV2s. Importantly, the new hydrophobic profile change lies within the second immunogenic epitope, (amino acid residues 113 to 139), of the nucleocapsid protein. These molecular data suggest that the lack of PCV2 virulence before 1970-1 may be intrinsic attribute of the three-dimensional structure of the nucleocapsid associated with surface-expressed epitope two. The corollary to this is that mutation(s) within ORF2 must have occurred after 1971. These mutation(s) altered the three-dimensional structure of arch-PCV2 into the con-PCV2 configuration and thus, a genomic virulence “factor” was inadvertently acquired by archival avirulent PCV2.

To test this hypothesis and to further investigate the biological significance of this novel archival ORF2 linear sequence, a cloned (c) and engineered (eng) arch-PCV2 was created from c-eng-con-

PCV2 by site-directed mutagenesis wherein the GCC-ACA-GCC codons (bases 1331-1339) of c-eng-con-PCV2 were replaced with ACA-GGG-AAC sequence of c-eng-arch-PCV2. The resultant Imp. Stoon 1010 PCV2a constructs both contain identical ORF1 (viral DNA replicase) sequence. These porcine kidney (PK) 15 cell-adapted and propagated PCV2 clones were tested for infectivity and virulence in gnotobiotic swine. The results of these various challenge experiments are reported here.

Objectives: ***The critical virulence determinant of swine-virulent PCV2 resides in a linear 3-4 amino acid region in the center of the second immunogenic epitope of PCV2 nucleocapsid protein.*** The overall goal (objective) of this grant proposal is to demonstrate that cloned engineered archival (c-eng-arch-) PCV2 is avirulent in piglets subsequently challenged with wild-type PCV2 and that this virus can be developed as an MLV candidate vaccine for the PCVDs. Two experimental aims address this experimental goal:

Aim 1: Conduct co-infection challenge experiments with c-eng-arch-PCV2 and known virulent wild-type (Stoon 1010) PCV2 in gnotobiotic swine to determine if c-eng-arch-PCV2 promotes PMWS caused by Stoon 1010 (PCV2a) and,

Aim 2: Conduct a limited vaccination-challenge experiment with c-eng-arch-PCV2 to develop "proof of concept" that this virus may serve as either an "improved" killed virus vaccine or more importantly, a modified-live-virus (MLV) vaccine for prevention of PCVDs.

Materials and Methods:

Part 1 (*In vitro* studies):

PCV2 viruses: Wild type (WT) PCV2a Imp. Stoon 1010 (PCV2/OSU) virus con-tained in a PMWS-affected gnotobiotic pig tissue homogenate (10%, w/v), *in vivo* pass three (PCV2/OSUp3) was used as source viral material for cloning experiments. *In vitro* working pools of PCV2/OSUp4 (2.73×10^7 DNA copies/ 100ng total DNA; 3.6×10^8 PK-infectious units $_{50}$) were derived by inoculation of tissue homogenate onto glucosamine-treated PCV-free porcine kidney (PK) 15 cells with subsequent *in vitro* passes in PK15 cells as described elsewhere. As well, PK15-adapted PCV2s were propagated and titrated in adenovirus-transformed porcine alveolar macrophage 3D4/2 cells (ATTC: CRL 2845).

Cloning PCV2/OSUp3: PCV2a from PK15 cell-adapted PCV2/OSUp3 con-taining 2.4×10^8 PCV2 DNA copies/100ng total DNA (4.3×10^8 PK15 cell virus infectious units$_{50}$) was cloned into pGem-Clone 14 (F055392) by standard methods. Aliquots of this virus was engineered (eng) to contain the codons for the arch-PCV2 nucleocapsid amino acid sequence (Thr-Gly-Asn) in the base positions 1331-1339 of ORF2 using site-directed mutagenesis at Department of Agriculture, Northern Ireland (DANI), now Agri-Food and Biosciences Institute (AFBI) by Dr. Brian Meehan. Following site-directed mutagenesis, the presence of the 1339 ACA (Thr) CGG (Gly) AAC (Asn) 1331 archival and 1339 GCC (Ala) ACA (Thr) GCC (Ala) 1331 contemporary genotypes were confirmed by complete nucleotide sequencing of each clone. Clone 1010L of c-eng-con-PCV2 con-tained the codons for the contemporary Ala-Thr-Ala amino acid sequence; clone FM8 of c-eng-arch-PCV2, contained codons for the Thr-Gly-Asn amino acid sequence. Each was selected for further *in vitro* and *in vivo* analyses.

Propagation of cloned PCV2s in PK15 cells and 3D4/2 macrophages: Viral stocks of c-eng-con-PCV2 and c-eng-arch-PCV2 containing 1.12×10^6 and 2.12×10^6 PCV2 DNA copies per 100ng of total DNA respectively were prepared in separate PK15 cultures following transfection with *Eco RI* excised cloned PCV2 DNAs. Thereafter, these viruses were propagated in PK15 cells with glucosamine. For *in vivo* studies in gnotobiotic piglets, the sixth *in vitro* (PK15 cell) pass of c-eng-arch-PCV2 and c-eng-con-PCV2 inocula were derived from 5.0 ml of PK15 extract containing

$2.12x10^6$ c-arch-PCV2 DNA copies/100ng total DNA arch-PCV2 and 1.12^6 PCV2 DNA copies/ 100ng total DNA of c-con-PCV2 respectively.

Immunohistochemistry (IHC): Monoclonal 2B1 was used to identify PCV2-nucleocapsid protein in tissue sections and in virus-infected monolayers. Mono-clonal D10 was used to detect the viral DNA replicase protein, the ORF1 gene product of PCV2. The general procedures used to stain ethanol-fixed, paraffin-embedded five-micron sections of porcine tissues have been reported previously.

***In situ* hybridization (ISH) for PCV2 DNAs:** *In situ* hybridization (ISH) was used to detect c-eng-arch-PCV2, c-eng-con-PCV2 and PCV2/OSUp4 viral DNAs in tissues. A DIG-labeled PCV2-specific probe was produced by PCR amplification of PCV2 DNA with the following primers: Forward: 5´ CGG ATA TTG TAA TCC TGG TCG 3´ and reverse: 5´ CTG TCA AGC GTA CCA CAG TCA 3´ using a commercially available kit (Roche Diagnostics Corporation, Indianapolis, IN). The DIG-labeled probe that hybridized to target PCV2 DNAs was detected by binding with horseradish peroxidase-conjugated anti-DIG antibody using diamino-benzidine (DAB) as developing reagent (Vector Laboratories, Burlingame CA). Slides were counterstained, dehydrated, mounted and examined by light microscopy.

Quantitative (q) PCR for PCV2 DNAs: Tissue samples from selected PCV2-infected piglets were homogenized in Hank's balanced salt solution (HBSS) buffered with HEPES containing penicillin, neomycin and streptomycin to form a 10% (w/v) solution. Total DNAs were extracted from 100µl of homogenized tissue, porcine sera and tissue culture lysates using the *Qiaquick*^{R} DNA extraction kit for blood and tissues as per the manufacturer's instructions (Quiagen Inc. USA, Valencia CA)**.** The qPCR was performed on a Roche Lightcycler 480 with *SYBrgreen master mix* (Roche Diagnostic Corporation, Indianapolis, IN) based upon primers and conditions as follows: Activation 95C for 10 min, one cycle; 40 cycles of amplification (95C 10s; 55C 15s; 72C 17s;); melting curve 65C - 97C; 42C for 60s. The primer set used (ATA AAA ACC ATT ACG AAG TGA TA and GCT CTC TAT CGG AGG ATT AC) is PCV2-specific. Cloned PCV2-containing plasmids were used to construct standard curves for both c-eng-arch-PCV2 and c-eng-con-PCV2.

Validation of the qPCR assay was accomplished by a comparison of qPCR values to quantitative recovery of infectious virus on PK15 cells. For this, stored (-70C) samples of liver (n=7), pooled axillary and inguinal lymph nodes (n=3) and bronchial lymph nodes (n=4) from piglets randomly selected from various challenge groups below were homogenized, serially diluted (ten-fold) in HBSS and inoculated onto PK15 cells in 96 well plates. After 24 hrs, infected cells were treated with 100mM glucosamine hydrochloride and incubated for an additional 72 hrs. Monolayers were preserved in 80% acetone: 20% PBS (v/v) fixative, stained by IHC and fifty percent titer endpoints were calculated.

Restriction endonuclease digestion analysis: DNA was extracted from porcine tissues, c-eng-arch-, c-eng-con-PCV2-infected PK cell lysates as described above and amplified (30 replication cycles) using the same primer sets used for production of ISH probe. The reaction mixture consisted of 50% 2x *HotStar Master Mix*^{R} (Roche Diagnostics Corporation, Indianapolis, IN), 0.2µM of each primer, template and DNase-free sterile water to a total volume of 50µl. Twenty-eight microliters of the resultant reaction product was digested BsaWI for three hours. Both cut and un-cut PCR amplicons were analyzed on a 1.5% agarose gel in Tris-acetate EDTA (TAE) buffer.

Part 2 (*in vivo* studies):

Gnotobiotic piglets: Sixty-two (62) gnotobiotic pigs from all or portions of seven litters were derived into sterile conditions via Cesarean section from specific-pathogen-free sows as described previously.

Each challenge and treatment group was housed separately within separate isolation units. All piglets in all experimental challenge groups were terminated at 33-36 days of age or earlier if moribund. Gross lesions were recorded and replicate samples for histopathology, virus isolation and/or direct and quantitative (q) PCR assays were collected. Prior to and at the conclusion of the experiments, each isolation unit (food, feces and cages) was cultured for aerobic and anaerobic bacterial growth to confirm the microbe-free status of the experiments. The experimental designs used for each set of *in vivo* studies are separately described below.

Infected with c-eng-arch-PCV2 only: Piglets of Group A (n=6) were inoculated intranasally (IN) with 5.0 ml c-eng-arch PCV2, 2.5 ml per nostril containing $2.12x10^6$ c-eng-arch-PCV2 DNA copies/100ng total DNA. Piglets of Group B (n=4) were similarly inoculated with 5.0 ml c-eng-con-PCV2 containing 1.12^6 c-eng-con-PCV2 DNA copies per 100ng total DNA. Piglets of Group C (n=5) were inoculated IN with 1.0 ml tissue homogenate PCV2/OSUp4 containing $3.6x10^8$ infectious units of virus per ml. Control piglets of Group D (n=3) were not inoculated with PCV2 but rather received sterile saline solution IN.

Infected with c-eng-arch-PCV2, immune stimulation: The general design for this series of experiments was as follows: Piglets were infected with various viruses at three days of age and then immune stimulated with two injections of keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant (KLH/ICFA) at 5 and 12 days of age. Piglets of Group A1 (n=3) were inoculated with 2.0 ml c-eng-arch-PCV2 and then immune stimulated with KLH/ICFA. Piglets of Group B1 (n=4) were infected with c-eng-con-PCV2 and immune stimulated with KLH/ICFA. Fourteen (14) piglets of Group C1 were infected with PCV2/OSUp4 and immune stimulated with KLH/ ICFA. Piglets of Group D1 (n=2) were given sterile saline IN and then immune stimulated with KLH/ICFA.

Sequential infection with c-eng-arch-PCV2 and PCV2/OSUp4: For this experiment, c-eng-arch-PCV2 containing 1 x $10^{5.5}$ infectious units of c-eng-arch-PCV2/ml (1.2 x 10^6 PCV2 DNA copies per 100ng total DNA) was inoculated IN into four (4) gnotobiotic piglets, 0.5 ml per nostril, at 3 days of age. One week later, PCV2 challenge virus (PCV2/OSUp4), a 10% (w/v) tissue homogenate containing 5 x 10^8 infectious units per ml was given by the intranasal (IN) route. Two piglets were similarly infected with PCV2/OSUp4 alone and two separately housed piglets served as uninfected controls.

Vaccination with c-eng-arch-PCV2, challenge with PCV2/OSUp4 and immune stimulation: Nine (9) piglets were inoculated intramuscularly (IM) with PK-5 cell-passed eng/arch/PCV2 (10^5 DNA copies/ng of total DNA equivalent to 10^5 infectious units of virus) intramuscularly (IM) at two days of age. At 16 days of age, piglets were challenged IN with PCV2/ OSUp4. At 21, 28 days of age, piglets were immune stimulated with KLH/ICFA. As infection controls for this experiment, two piglets were inoculated with PCV2/OSUp4 at 16 days of age and immune stimulated as above. Two piglets served as unvaccinated control piglets.

Statistics: Except for viral titration data, statistical analysis of the data was performed by the OSU Center for Biostatistics. Analysis of variance (ANOVA) was used to assess the log-transformed qPCR data. A Tukey method was used to adjust for multiple comparisons. Histological findings were assessed with a Fisher's exact test (both one- and two-sided) based on the number of piglets in each outcome category by infection group. Statistical analyses on the PCV2 viral titration data were performed as follows: The normal distribution of the titer data from each organ was confirmed by an F-test. The P values for these data were calculated using a one-tailed, two-sample T-test assuming unequal variances.

Results:

PCV2 and cloned PCV2 viruses: The original PCV2 clone(s) of reconstructed archival and contemporary (PCV2a, Stoon 1010) PCV2 was prepared in the Department of Agriculture, Northern Ireland (DANI) by Dr. B. Meehan and sent to the OSU laboratory as plasmid(s). PCV2 DNAs were excised from plasmids using *Eco RI* and viral DNAs were transfected into PK15s by electroporation at OSU.

Two PK15-adapted clones of arch-PCV2 were evaluated at OSU. The first clone (designated x-c-eng-arch-PCV2) contained a random single nucleotide mutation of lysine (L)-58 (AAG) to G-58 (GAG) in the ORF2 genome. As well nucleotide substitution in bases 1331 and 1339 was only partially successful in that only 7 of 9 bases conformed to the desired archival sequence. Finally, x-c-arch-PCV2 replicated poorly in PK15s. For these reasons, x-c-eng-arch-PCV2 was not studied further. A second clone of arch-PCV2 that contained 3.04×10^6 PCV2 DNA copies per 100ng of total DNA was evaluated. Sequencing of this cloned viral DNA product after site-directed mutagenesis to correct the lysine-58 change demonstrated that cloned-engineered-archival-PCV2 (c-eng-arch-PCV2) was sequence-identical to contemporary virulent PCV2 (Stoon 1010) at L-58 and contained all nine altered base sequence changes identified in the initial sequencing experiments with archival DNAs. The c-eng-arch-PCV2 was excised from plasmid by Eco RI digestion and transfected into PK15 cells; this virus replicated well in PK15s and infectious viral stocks of c-eng-arch-PCV2 containing 2.12×10^6 PCV2 DNA copies/100ng of total DNA was prepared for subsequent studies.

A clone of virulent contemporary PCV2, prepared from PCV2a (Stoon 1010), was also constructed and then sequenced. The ORF2 product was sequence-identical to c-eng-srch-PCV2 except for the nine bases, (positions 1131 through 1339) and was also identical to contemporary virulent PCV2 (PCV2/OSUp4). After transfection, PK15 viral stock(s) of cloned-engineered-contemporary-PCV2 (c-eng-arch-PCV2) were prepared as described above; this pool contained 1.12×10^6 PCV2 DNA copies/100ng of total DNA.

Virus titrations and qPCR: The qPCR assay for PCV2 DNA copy numbers was validated by comparison to the quantitative recovery of infectious contemporary PCV2 on PK15 cells. Linear regression of viral DNA copy number (qPCR) versus viral titer data for PCV2-positive samples resulted in an r^2 value of 0.7265, $p<0.0005$ with a slope of 2.4×10^{-3}.

***In vitro* IHC:** Uninfected control PK15 monolayers did not stain with either nucleocapsid-specific monoclonal antibody 2B1 or viral DNA replicase protein monoclonal antibody D10. All cloned and uncloned PCV2 viruses reacted with D10 monoclonal antibody, a finding consistent with the molecular data that identified the ORF1 (viral DNA replicase) gene as sequence-identical in all viruses. As expected, both PK15-origin contemporary PCV2 strains as well as virulent PCV2 recovered from PCV2/OSUp3-infected piglets reacted strongly with monoclonal 2B1; PK15-origin c-eng-con-PCV2 also reacted with this monoclonal reagent. In contrast, PK15 monolayers containing c-eng-arch-PCV2 did not stain with monoclonal 2B1, even though replicates of these same monolayers reacted with the viral DNA replicase protein, monoclonal D10.

Restriction endonuclease digestion (data not shown): The restriction endonuclease, BsaWI cleaved both c-eng-arch-PCV2-DNA and x-c-arch-PCV2-DNA but not c-eng-con-PCV2-DNA or PCV2/OSUp4-DNA. Prior to digestion, the amplified PCR product from each virus was approximately 475 base pairs long. After BsaWI digestion of c-eng-arch-PCV2 DNA a product one-half the length of the original remained. Viral DNAs recovered from liver of piglets infected with c-eng-arch-PCV2 and c-eng-con-PCV2 behaved similarly to the PK15-origin viral DNAs. BsaWI DNAse digestion thus confirmed that both PK15- and liver-origin c-eng-arch-PCV2 DNAs were identical.

Replication of cloned PCV2s in 3D4/2 macrophages: All *in vivo* experiments (described below) indicate that c-eng-arch-PCV2 is avirulent for swine, even though this virus, like it's virulent counterparts, replicates in cells of monocyte-dendritic cell morphology. As noted, c-eng-arch-PCV2 readily adapted to growth in PK15 (epithelial) cells; levels of viral DNAs achieved with c-eng-arch-PCV2 (0.14 to 2.1 x 10^6 DNA copies per 100 ng total DNA) were equivalent to those achieved with c-eng-con-PCV2 (6.7 to 1.2 x 10^6 DNA copies per 100 ng total DNA). To determine if the lack of virulence exhibited by c-eng-arch-PCV2 could be associated with a relative inefficiency of viral replication in porcine macro-phages, replicate cultures of 3D4/2 cells were inoculated with equivalent amounts sixth *in vitro* pass of PK15-origin cloned viruses. Viral DNA levels were assessed by qPCR through three-24 hour replication cycles in these cell monolayers. The quantitative PCR PCV2 DNA data for the first 24-hour replication cycle are summarized in Table 6. When compared to both PCV2/OSUp4 and c-con-eng-PCV2, c-eng-arch-PCV2 cultures contained roughly two log-base ten fewer viral DNAs per 100 ng of total cell DNA (Table 6). This trend (lower values for c-eng-arch-PCV2 than for the two contemporary virulent viruses) continued through the 48- and 72-hour replication cycles (data not shown).

Clinical assessment and gross findings: Piglets were monitored for signs of clinical illness throughout the course of these experiments. In piglets that were immune stimulated with KLH/ICFA, regardless of their infection status, mild-to-moderate peripheral lymphadenopathy associated with mineral oil adjuvant accumulations in axillary and superficial and deep inguinal lymph nodes was a regular occurrence.

Virulence trials (Table 2): Gross findings from all single agent challenge experiments are summarized in Table 2. Neither clinical signs of PMWS nor gross lesions compatible with PCV2 infection were present in the uninfected control piglets of Groups D and D1. Aside from mild bronchial lymphadenopathy in one of four piglets given c-eng-con-PCV2 alone (Group B) and three of five piglets inoculated with PCV2/OSUp4 alone (Group C), no evidence of PMWS was seen. Six of 14 piglets inoculated with PCV2/OSUp4 and then immune stimulated developed PMWS (Group C1); the remaining piglets in that group developed bronchial lymphadenopathy characteristic of subclinical PCV2 infection and/or mild PMWS. One of four piglets infected with c-eng-con-PCV2 (Group B1, Table 1) and immune stimulated with KLH/ICFA exhibited mild PMWS with ascites. No gross evidence of PCV2 infection was seen in piglets infected with c-eng-arch-PCV2 (Groups A and A1) whether or not immune stimulation with KLH/ICFA was used.

Potentiation/vaccination experiments (Table 3): Disease outcomes from the dual agent challenge and vaccination/challenge experiments are summarized in Table 3. To determine if c-eng-arch-PCV2 had potentiating disease effects for virulent contemporary PCV2, four piglets (Group A) actively infected with c-eng-arch-PCV2 were challenged with virulent PCV2/OSUp4 as described elsewhere; no gross or histologic evidence of PMWS was seen. Two piglets (Group B) challenged with virulent PCV2/OSUp4 alone remained clinically asymptomatic, as did the two uninfected controls of Group C.

To determine if c-eng-arch-PCV2 could function as a candidate modified-live vaccine (MLV) product for prevention of PMWS, nine piglets were "vaccinated" with an intramuscular injection of c-eng-arch-PCV2 at two days of age (Group A1). Two weeks later, all were challenged with virulent contemporary PCV2. At termination, neither lymphadenopathy nor other evidence of PMWS was seen. Serum PCV2 DNA copy numbers per 100 ng total DNA in these vaccinated-and- challenged piglets correlated to clinical outcome in that serum DNA copies in piglets after c-eng-arch-PCV2 vaccination and prior to virulent PCV2 challenge were low (5.3 x 10^3). Two weeks after challenge, PCV2 DNA copy numbers increased only slightly (1.2 x 10^4).

Histopathology: In general, histologic changes associated with subclinical PCV2 infection or PCV2 infection manifest as PMWS in gnotobiotic piglets paralleled gross findings above. Histologic evidence for PCV2 infection in c-eng-arch-PCV2-infected piglets was sought. The only histologic changes compatible with subclinical infection in these piglets were mild germinal center formation in the bronchial lymph nodes, paracortical T cell hyperplasia and rare syncytial giant cells in peripheral lymph nodes, particularly in the KLH/ICFA-immunized piglets.

***In situ* hybridization (ISH):** Tissue sections from c-eng-arch-PCV2-infected, c-eng-con-PCV2-infected and PCV2/OSUp4-infected were recognized by the ISH probes regardless of disease status; the controls were ISH-negative. Others have reported ISH data obtained from PMWS-positive and PMWS-negative PCV2-infected swine. In general, our ISH data agreed with these author's findings in that the relative abundance of ISH-positive mononuclear cells and syncytia (lymphoid tissues) and hepatocytes in PMWS-affected piglets were substantially more abundant in sections than were positive signals in tissues examined from subclinically infected piglets.

Of particular interest were the ISH stains from tissues collected from c-eng-arch-PCV2-infected piglets. Bronchial lymph nodes from these animals had small clusters of ISH positive cells in dendritic cells of the follicles only. Similar ISH-positive mononuclear cells were sparcely scattered within splenic sinuses and throughout peripheral and mesenteric lymph nodes, tonsil and ileal Peyer's patches. The ISH-positive monocuclear cells were only lightly positive with DAB reaction product. Liver and other parenchymal tissues from all c-eng-arch-PCV2-infected piglets were PCV2 DNA-negative by ISH. Statistical analysis of ISH data was generated from tissue section scans wherein ISH-positive pixels per mm^2 were compared using the Fisher exact test. In the peripheral lymph nodes there were fewer number of positive pixels per mm^2 in c-eng-arch-PCV2-infected when compared to the number of ISH-positive pixels identified in either the PCV2/OSUp4-infected ($p<0.006$) or c-eng-con-PCV2-infected peripheral lymph nodes ($p<0.03$). Conversely, there was no significant difference in the number of positive pixels/mm^2 in the c-eng-con-PCV2-infected versus PCV2/OSUp4-infect-ed sections of peripheral lymph nodes ($p>0.28$).

Tissue viral DNAs by qPCR: The validated qPCR assay for PCV2 DNA copy levels in sera and various tissues were performed in groups of piglets and is summarized in Tables 4 and 5. Levels of PCV2 DNAs per 100ng total DNA in tissues from c-eng-arch-PCV2- and c-eng-con-PCV2-infected groups ranged between 10^3 and 10^6 copies of PCV2 DNA per 100ng total DNA for the bronchial lymph nodes; there was no statistically significant difference ($p>0.90$) between these groups in this tissue (Table 4). Bronchial lymph nodes from PCV2/OSUp4-infected piglets of Groups C1 had qPCR values of greater than 10^6 copies of PCV2 DNA per 100 ng of total DNA; these values were statistically significant when tested against c-eng-arch-PCV2 ($p<0.003$) or c-eng-con-PCV2 ($p<0.001$). Splenic PCV2 copy numbers (data not shown) reflected trends established in bronchial lymph nodes in that DNA copy numbers in PCV2/OSUp4-infected piglets were greater ($p<0.005$) than those in the c-eng-con-PCV2-infected piglets and in piglets inoculated with c-eng-arch-PCV2 ($p<0.0001$). The liver is the target organ that fails in gnotobiotic piglets with PMWS and both DNA copy numbers and recoverable infectious virus in this organ reflect this fact. The liver PCV2 DNA levels in the liver from c-eng-arch-PCV2-infected piglets were $<10^3$ copies of DNA compared to values (10^3-10^6 copies of PCV2 DNAs per 100ng of total DNA), obtained from the livers of the other two PCV2 infection groups. There was a statistically significant difference across the three tested piglet groups ($p<0.001$) with respect to hepatic viral DNA copy values. The PCV2/OSUp4-infected piglets had hepatic viral DNA copy numbers higher than either c-eng-con-PCV2 or c-eng-arch-PCV2 piglet groups ($p<0.002$ and $p<0.0001$ respect-ively). The c-eng-arch-PCV2-infected pig-lets had hepatic PCV2 viral DNA copy numbers higher than the mean values obtained from the c-eng-arch-PCV2-infected piglets ($p<0.002$).

Serum PCV2 DNA qPCR values for piglets infected with cloned PCV2 viruses are summarized in Table 5. The group values obtained support the data generated from tissue homogenates in that a hierarchy of mean terminal PCV2 DNA copy numbers was established as follows: Uninfected controls = 0.0×10^0; c-eng-arch-PCV2 alone = 1.3×10^3; c-eng-arch-PCV2 and KLH/ICFA = 6.3×10^3; and c-eng-con-PCV2 and KLH/ICFA = 1.7×10^6 PCV2 DNA copies per 100 ng total DNA.

Discussion: Through retrospective serology using nucleocapsid protein as the ELISA antigen, others have shown that asymptomatic PCV2 virus(es) infections were present in swine populations for at least 25 years prior to the first reported case of PMWS. These serologic data are supported by the recovery of PCV2 DNAs from archived porcine tissues. For these studies, archival (arch) PCV2 sequences were recovered from swine tissues collected in 1970-1971 originally for an Aujesky's disease surveillance project in Northern Ireland; reconstruction of arch-PCV2 genome from these data was accomplished there. The ORF1 sequence (the viral DNA replicase protein) was essentially unchanged whereas the ORF2 that codes for the nucleocapsid protein contained a linear sequence difference of nine bases (three codons) in positions 1331 through 1339, corresponding to amino acid residues 133-135 of the nucleocapsid protein. This altered sequence lies entirely within the second immunogenic surface epitope (residues 113 to 139) of the nucleocapsid. The objective of the experiments reported here was to determine if the putative three amino acid change (Thr-Gly-Asn to Ala-Thr-Ala), identified by comparative ORF2 sequence analyses between the arch- and con-PCV2s, is critical for *in vivo* viral virulence. This was accomplished by direct *in vivo* comparison of the biological behavior of an arch-PCV2 to that of virulent con-PCV2. Both viruses were engineered from cloned virulent PCV2a (PCV2/OSUp4) by substituting the archival sequence at bases 1331 through 1339 in one clone (c-eng-arch-PCV2) and comparing it to a clone containing the contemporary 1331 to 1339 sequence (c-eng-con-PCV2) in those same positions.

Sequence analysis of our first cloned engineered archival PCV2 (x-c-eng-PCV2) indicated that cloning and engineering was partially successful in that seven of nine nucleotides between nucleotides 1331 and 1339 were altered. However, an additional random mutation from AAG to GAG (Lys-to-Glu), corresponding to amino acid 58 in the N-terminus of nucleocapsid protein was found in the ORF2 gene when the DNA was sequenced. Virulence data collected from x-c-eng-arch-PCV2 indicated that it is capable of replication *in vivo* but did not cause PMWS under conditions of either immune stimulation with KLH/ICFA or immune suppression with oral cyclosporine (data not shown). However, x-c-eng-arch-PCV2 did not replicate readily in PK15s and several *in vivo* passes in gnotobiotic swine were needed to produce appropriate volume/titer of challenge inocula for additional study. The poor *in vitro* performance of this first clone may be related to the additional L-to-G mutation in amino acid 58, located near the N-terminus of the nucleocapsid protein. To correct this mutation and to get a complete base sequence change in our region of interest, a perfect clone of arch-PCV2 was produced through site-directed mutagenesis of cloned PCV2/OSUp3, Stoon 1010 in which only the ACA (Thr) GGG (Gly) AAC (Asn) change was present. In these second generation constructs, all other codons for both the viral replicase and nucleocapsid protein were identical. Unlike x-c-arch-PCV2, these clones replicated well *in vitro* and for this reason, additional *in vitro* and *in vivo* studies were conducted with them.

In gnotobiotic piglets, levels of PCV2 in target tissues such as the lymphoid system and liver directly correlates to disease outcome. Infectious viral titers greater than 10^7-10^8 infectious units/gram of tissue correlate to development of PMWS; levels below this produce either mild PMWS or subclinical infection. The lack of overt PMWS in piglets inoculated with c-eng-con-PCV2 most likely reflect the low dose of challenge inoculum (roughly 10^6 versus 10^8 in PCV2/OSUp4) since the sequence of c-eng-co-PCV2 was identical to that of PCV2/OSUp4. Regarding the clone of greatest interest, (c-eng-arch-PCV2), *in vivo* infection data suggest that infection by this virus was restricted to lymphoid

tissues. Moreover, the low levels of viral DNA (both qPCR and ISH) in lymphoid tissues from this group of piglets indicate that this virus replicated less efficiently than did either c-eng-con-PCV2 or PV2/OSUp4. By ISH, both c-eng-con-PCV2 and c-eng-arch-PCV2 infect primarily mononuclear dendritic cells. Lack of ISH signal in sections of liver from piglets inoculated with c-eng-arch-PCV2 correlated to the low amounts of viral DNA present in this organ and to the lack of mononuclear inflammatory cell infiltrates as well. The latter is a hallmark of subclinical PCV2 infection in gnotobiotes. This was not due to intrinsic differences in the rate of DNA replication between these two viruses as both replicated to similar titers through six *in vitro* passes through PK15 cell monolayers (see Table 6).

All PCV2 viruses evaluated in this study replicated in PK15 cells; the infection cycle for each virus was complete before 24 hours post-infection, was not cytolytic and cytopathic effects (cpe) were not seen. These features of *in vitro* replication have been noted with other PCV2 isolates. In the absence of cpe, another method for detecting viral replication and titer endpoints is by staining infected monolayers with a monoclonal antibody that reacts with the viral DNA replicase. All viruses used in this study reacted with monoclonal D10 indicating that the viral replicase was identical for all viral constructs. This was not the case for monoclonal 2B1, a nucleocapsid-specific monoclonal. Only c-eng-con-PCV2 and PCV2/OSUp4 viral progeny reacted with this reagent, both *in vitro* and *in vivo.* This difference in 2B1 monoclonal reactivity was preserved through the multiple rounds of viral propagation *in vitro* that were necessary to produce the pools of inocula used in this study, indirectly indicating that the three-amino acid linear sequence characteristic of c-eng-arch-PCV2 is replication-stable. Monoclonal 2B1 neutralizes the *in vitro* infectivity of contemporary PCV2 strains and is widely used for diagnostic purposes to identify PCV2 nucleocapsid protein in PCVD-suspect tissue samples. Our data suggests that monoclonal 2B1 is specific for the second immunogenic epitope previously identified on the surface of the nucleocapsid protein. Finally for *in vitro* viral titrations, the IHC stain for the viral DNA replicase IHC underestimates the amount of infectious virus present in the monolayers (Krakowka S., unpublished, 2004). Moreover, since c-eng-arch-PCV2 does not react with 2B1, an alternative method for quantitation of virus was needed. Estimates of viral infectivity for both x-c-eng-arch- and c-eng-arch-PCV2 viruses were accomplished by a validated qPCR wherein PK15-infectious virus titers ($TCID_{50}$) was related to qPCR DNA levels by linear regression analysis.

One explanation for the apparent lack of pathogenicity and low tissue DNA copy numbers associated with c-eng-arch-PCV2 *in vivo* is inefficient packaging of virion progeny in macrophages specifically. For PCV2, the nucleocapsid N-terminus lies on the internal face of the capsomeres and interacts directly with packaged circular viral DNA. The nucleocapsid C-terminus has both structural and functional components and is primarily responsible for the self-assembly of capsomeres into the final icosahedral shape. These C-terminus surface epitopes also interact with the environment. The Thr-Gly-Asn to Ala-Thr-Ala difference between c-eng-arch-PCV2 and c-eng-con-PCV2 occurs in the C-terminus and may affect virion assembly by reducing the docking efficiency of c-eng-arch-PCV2 capsomeres. Some support for this proposed mechanism is seen in the *in vitro* titration data generated using porcine alveolar macrophage cells (CD4/31 cells), an *in vitro* equivalent of the primary tropic cell-type for PCV2 *in vivo*, macrophages and dendritic cells in lymphoid tissues. When PK15 and CD4/31 cells were inoculated with both cloned viruses and PCV2/OSUp4, the former at identical multiplicities of infection (0.01), both virulent and avirulent clones replicated to the same level in PK15 cells but c-eng-arch-PCV2 DNA levels were two log-base 10 levels lower than was c-eng-con-PCV2. This suggests that encapsidation inefficiency within virus-infected macrophages *in vivo* may explain the low titers of resultant c-eng-arch-PCV2 produced by these cells and thus the inability to induce PMWS in PCV2-susceptible gnotobiotic piglets.

A computerized NCBI blast search reveals that the specific three-amino acid change identified in arch-PCV2 does not occur in any published contemporary strains of PCV2. Both Imp. Stoon 1010

(PCV2/ OSUp4) and c-eng-arch PCV2 are members of genogroup PCV2a as defined by the European Consortium on Porcine Circovirus Diseases. The sequence variation(s) in ORF2 used by others to identify PCV2 genogroups are independent of the three amino acid region defined in these studies. In a recent study, Opriessnig compared two strains of contemporary virulent PCV2; neither of which caused PMWS in young conventional swine. In that study, nine discontinuous amino acid changes were identified in a PCV2 strain that appeared to be relatively avirulent in piglet challenge experiments. Cloned-eng-arch-PCV2 shares one amino acid change in common with Opressnig's low pathogenicity PCV2 strain. The Gly-to-Pro change along with an additional Thr-to-Pro change at amino acid 131 identified in the less pathogenic strain may have the same effects as does the Thr-Gly-Asn change of c-eng-arch-PCV2. Although proline and glycine are both non-polar residues, glycine has the smallest R-group of all amino acids and has the greatest flexibility about the long axis of the peptide chain. In contrast, proline has a large and inflexible R-group that confines it to a narrow range of angles within a peptide chain thus greatly influencing the direction/shape of the surrounding peptide. Two proline residues in close proximity without an intervening glycine residue creates a rigid structure that differs from the more "flexible" contemporary Ala-Thr-Ala pattern.

The lack of virulence of eng-arch-PCV2 *in vivo* suggests that c-eng-arch-PCV2 could be developed for use as a modified-live-virus (MLV) vaccine for prevention of PCVDs by taking advantage of the fact that immunogenic epitopes one and three of c-eng-arch-PCV2 are identical to those of both PCV2a and PCV2b geno-groups. Currently, a number of different killed PCV2 vaccines are used; all show remarkable efficacy both at reducing the incidence of PMWS in herds clinically affected with PCVD and also in preventing the emergence of PCVDs in PCV2-infected herds. One goal of the *in vivo* experiments reported here was to determine if an MLV approach to control of PCVDs is even feasible using a well-characterized induction method for PMWS (immune stimulation with KLH/ICFA) in the gnotobiotic piglet model of disease. A necessary preliminary experiment was to determine if sequential infections of gnotobiotic piglets with c-eng-arch-PCV2 and virulent PCV2/OSUp4 had similar to the promotional effects of PCV2a and PCV2b infections in gnotobiotic swine. This did not happen and these data further support the inherent avirulence (safety) profile of c-eng-arch-PCV2. The pilot vaccination and challenge experiment, complete with immune stimulation by KLH/ICFA infections appeared to prevent PMWS, likely by keeping PCV2 levels below that established for induction of PMWS in this model system. However, at the moment, given the unqualified success of killed vaccine products, it is unlikely that an MLV vaccine, administered in the usual circumstances, could materially improve on this record of efficacy and efficiency. In the future, there may be "niche" market for an MLV PCV2 product, particularly if the MLV virus becomes established in the herd as a subclinical and stable infection.

In summary, we have shown that a PCV2 clone containing an archival amino sequence within epitope two of the nucleocapsid is replication-competent but avirulent in PCV2-susceptible swine, in swine immune stimulated with KLH/ICFA (and in piglets co-infected with virulent PCV2. Collectively, the data demonstrate that a nucleocapsid surface moiety on or within epitope two is the critical viral determinant needed to generate PMWS by virulent contemporary PCV2 strains. Moreover, these data support the hypothesis that archival avirulent strain(s) of PCV2, circulating in swine populations before 1970 are responsible for the generation of PCV2-specific antibodies in these pigs. We suggest that mutation(s) within epitope two, specifically in amino acid residues 131 to 133, altered the virulence potential of PCV2 without significantly affecting the usual parameters of viral infectivity and transmission amongst swine. As intensive management practices including early segregated early weaning and early vaccinations were widely implemented in swine production in the early 1990's, conditions became conducive for expression of the full virulence potential of contemporary PCV2 as PMWS and other PCVDs.

Table 1: Titration of infectious virus in porcine kidney (PK) 15 cells and porcine al-veolar macrophage cell line (CD4/31 cells) as determined by quantitative polymerase chain reaction (qPCR) for PCV2 viral DNAs.

PCV2 strains	porcine kidney (PK) 15 cells	porcine alveolar macrophage (3D4/2) cells [a, b]
c-eng-arch-PCV2:		
in vitro passes 3-5	3.3, 4.4, 1.4 x 10^5 DNA copies per 100 ng total DNA	nd
in vitro pass 6	2.1 x 10^6 DNA copies per 100 ng total DNA	7.1 x 10^5 DNA copies per 100 ng total DNA
c-eng-con-PCV2:		
in vitro passes 3-5	8.9, 16.0, 6.7 x 10^5 DNA copies 100 ng total DNA	nd [c]
in vitro pass 6 [d]	1.2 x 10^6 DNA copies per 100 ng total DNA	3.1 x 10^7 DNA copies per 100 ng total DNA
PCV2/OSUp4: [e, f]	2.7 x 10^7 DNA copies per 100 ng total DNA	3.6 x 10^7 DNA copies per 100 ng total DNA

[a] The 3D4/2 cells also contained noninfectious fragments of PCV2 DNAs, (<1.0x10^2 DNA copies per 100 ng total cell DNA).

[b] 3D4/2 cells were inoculated with PK15-origin PCV2 (*in vitro* pass 6) at a multi-plicity of infection (MOI) of 0.10.

[c] nd = not done.

[d] The PK15 pool of c-eng-arch-PCV2, *in vitro* pass 6 was used as the test modified-live virus (MLV) candidate PCV2 virus.

[e] A 10% (w/v) PCV2/OSUp4 tissue homogenate, used to infect 3D4/2 cells was propagated *in vitro* for 6 passes in PK15 cells.

[f] PCV2/OSUp4 10% (w/v) tissue homogenate was used to infect PK15 cells.

Table 2: A summary of gross and clinical findings in gnotobiotic piglets infected with various strains of porcine circovirus type 2 (PCV2) with or without immune stimulation by keyhole limpet hemocyanin (KLH/ICFA) emulsified in incomplete Freund's adjuvant.

Piglet infection Groups	peripheral (adjuvant-related) lymphadenopathy	bronchial lymphadeno-pathy	early death (PMWS), edema, icterus interstitial pneumonia
Piglets infected with various PCV2s alone:			
Group A (n=6) [a]: Infected with c-eng-arch-PCV2 alone:	0/6	0/6	0/6
Group B (n=4): Infected with c-eng-con-PCV2 alone:	0/4	1/4	0/4
Group C (n=5): Infected with PCV2/OSUp4 alone:	0/5	3/5	0/5
Group D (n=3): Uninfected controls:	0/3	0/3	0/3
Piglets infected with various PCV2s and immune stimulated with KLH/ICFA[b]:			
Group A1 (n=3): Infected with c-eng-arch-PCV2:	3/3	0/3	0/3
Group B1 (n=4): Infected with c-eng-con-PCV2:	4/4	1/4	1/4 (ascites)
Group C1 (n=14): Infected with PCV2/OSUp4:	14/14	14/14	6/14
Group D1 (n=2): Uninfected controls:	2/2	0/2	0/2

[a]Number of piglets (parentheses) per challenge group. [b]KLH/ICFA immunizations were given on days 5 and 12 of age.

Table 3. The effects of intramuscular (IM) vaccination with c-eng-arch-PCV2 and sub-sequent infection with PCV2/OSUp4 upon the induction of PMWS in gnotobiotic piglets with or without immune stimulation by keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant. (KLH/ICFA).

Piglet Group	Outcome of Disease: stable subclinical infection	mild PMWS	overt PMWS
Potentiation of PMWS by c-eng-arch-PCV2:			
Group A (n=4)[a], Infected with c-eng-arch-PCV2 and PCV2/OSUp4:	4	0	0
Group B (n=2), Infected with PCV2/OSUp4 at 16 days of age	2	0	0
Group C (n=2), Uninfected controls:	2	0	0
Vaccination with c-eng-arch-PCV2; challenge with PCV2/OSUp4; immune stim- ulation with KLH/ICFA: [b]			
Group A1 (n=9): Vaccinated IM with c-eng-arch-PCV2 and infected with PCV2/OSUp4:	9	0	0
Group B1 (n=2), vaccinated IM with c-eng-arch-PCV2:	2	0	0
Group C1 (n=2), Uninfected controls:	2	0	0

[a] The number (in parentheses) is the number of piglets per experimental group.

[b] Piglets were immune stimulated with KLH/ICFA five and 12 days after chal-lenge with PCV2/OSUp4.

Table 4: A summary of PCV2 DNA levels determined by qPCR in selected tissues from piglets inoculated with various PCV2 strains and immune stimulated by two injections with keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant (KLH/ICFA).

Piglet Infection Groups	Bronchial Lymph Nodes $<10^3$	$10^3 – 10^6$	$>10^6$	P value: (A1 vs B1)	Liver $<10^3$	$10^3 – 10^6$	$>10^6$	P value: (A1 vs B1)
Group A1: Infected with c-eng-arch-PCV2 and immune stimulated with KLH/ICFA[a]:								
	0/3[b]	3/3	0/3	0.001	3/3	0/3	0/3	0.0001
Group B1: Infected with c-eng-con-PCV2 and immune stimulated with KLH/ICFA:								
	0/4	3/4	1/4	0.0009	0/4	4/4	0/4	0.001
Group C1: Infected with PCV2/OSUp4 and immune stimulated with KLH/ICFA:								
	0/3	0/3	3/3		0/3	3/3	0/3	
Group D1: Uninfected control piglets immune stimulated with KLH/ICFA:								
	0/2	0/2	0/2		0/2	0/2	0/2	

[a] KLH/ICFA immunizations were given on days 5 and 12 of age.

[b] Incidence = number of positives (numerator) over number tested (denominator).

Table 5: A summary of PCV2 serum DNA levels [a] collected in gnotobiotic piglets infected with various PCV2s.

Piglet Groups	Pre-inoculation sera	Terminal sera
Group 1 (n=6), infected (or vaccinated) with c-eng-arch-PCV2:		
mean	0.2×10^0	1.3×10^2
range	$0.0 - 1.2 \times 10^1$	$0.0 - 7.8 \times 10^2$
Group 2 (n=6), infected with c-eng-arch-PCV2 and immune stimulated, KLH/ICFA:		
mean	0.0×10^0	6.3×10^3
range	$0.0 - 0.0 \times 10^0$	$0.02 - 21.8 \times 10^3$
Group 3 (n=5); infected with c-eng-con-PCV2 and immune stimulated, KLH/ICFA:		
mean	0.4×10^0	1.7×10^6
range	$0.0 - 2.4 \times 10^1$	$0.0002 - 7.9 \times 10^6$
Group 4 (n=4); infected with PCV2/OSUp4 and immune stimulated, KLH/ICFA:		
mean	0.1×10^0	2.5×10^5
range	$0.0 - 4.8 \times 10^1$	$0.05 - 6.1 \times 10^5$
Group 5 (n=3); uninfected controls:		
mean	0.0×10^0	0.0×10^0
range	$0.0 - 0.0 \times 10^0$	$0.0 - 0.0 \times 10^0$

[a] Data expressed as the number of copies of PCV2 DNA per 100 ng total DNA.

[b] KLH/ICFA immunizations were given 5 and 12 days after PCV2 infection.