Title: Pathogenicity of the ORF3 gene-silence mutant of type 2 porcine circovirus in pigs: a study towards the development of a marker vaccine – **NPB #06-006**

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II: Industry Summary: Porcine circovirus type 2 (PCV2) currently is an economically important swine pathogen. Although several vaccines against PCV2 are available, it is important to understand the mechanism of PCV2 pathogenesis and develop improved vaccines. The open reading frame (ORF) 3 of porcine circovirus type 2 (PCV2) reportedly encodes a novel protein that is involved in pathogenesis, and that an ORF3-deletion PCV2 mutant is reportedly attenuated in mice. These reports have not been independently confirmed, and the results from the published mice pathogenesis study are difficult to interpretate. In this study, we attempted to verify the published reports in the relevant pig model and to assess whether or not the ORF3 can be used for PCV2 vaccine development. By using an anti-peptide ORF3-specific rabbit antibody, we could not detect the expression of ORF3 protein in PCV2-infected cells. Furthermore, when pigs, the natural host of PCV2, were inoculated with a ORF3-null PCV2 mutant, we did not find significant difference in the average scores of gross or histological pathological lesions between pigs inoculated with the ORF3-null mutant and pigs inoculated with wild-type PCV2. Thus, the data from this study do not support the conclusion of previous published reports, and thus the ORF3-null PCV2 cannot be used as a vaccine candidate. ***Contact information****: E-mail: xjmeng@vt.edu. X.J. Meng, M.D., Ph.D., Professor of Virology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA.*

III: Scientific Abstract:

The open reading frame (ORF) 3 of porcine circovirus type 2 (PCV2) reportedly encodes a novel protein that is involved in apoptosis. To characterize the role of ORF3 in PCV2 replication and pathogenesis, we first

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generated an anti-peptide ORF3-specific rabbit antibody and attempted to detect ORF3 protein expression by IFA and western blot from PCV2 infected cells. After repeated attempts, we could not detect any evidence of ORF3 protein expression in PCV2 infected cells, and thus could not confirm the earlier reports by Kwang's group. To evaluate the effect of ORF3, if any, on PCV2 replication and pathogenesis *in vivo*, we created an ORF3-null PCV2 mutant by site-directed mutagenesis. The ORF3-null mutant (muPCV2) infectious DNA clone and virus from transfected cells can initiate PCV2 infection when inoculated into pigs, indicating that the ORF3 is dispensable for PCV2 replication *in vivo*. Since the ORF3-deficient PCV2 was reportedly less pathogenic than PCV2 in BALB/c mice, in this study we compared the pathogenicity of an ORF3-null PCV2 mutant (muPCV2) and the wild-type PCV2 in the natural host, pigs. Thirty-one pigs were divided into 3 groups of 11, 10, and 10 each. The 11 pigs in group 1 were each inoculated with PBS buffer as negative controls, 10 pigs in group 2 were each intramuscularly inoculated with 200 µg of muPCV2 infectious DNA clone, and 10 pigs in group 3 each with 200 µg of PCV2 infectious DNA clone. Blood was collected prior to inoculation and weekly thereafter, and tested for PCV2 antibodies by ELISA and serum viral DNA loads by quantitative PCR. All pigs were necropsied at 35 days post-inoculation (DPI) and various tissues were collected and analyzed for gross and microscopic lesions. The results showed that there was no significant difference in the average scores of gross or histological pathological lesions between pigs inoculated with the ORF3-null PCV2 mutant and pigs inoculated with the wild-type PCV2, although pigs inoculated with muPCV2 did have a delayed appearance of seroconversion, and decreased serum viral DNA loads. Thus, the data from this study do not fully support the conclusion of the published report that ORF3-deficient PCV2 is less pathogenic in mice. Consequently, the use of ORF3-null PCV2 as a vaccine candidate is not justified.

IV: Introduction:

Porcine circovirus (PCV) was first discovered in 1974 and is a member of the family *Circoviridae* (20). The original virus, designated porcine circovirus type 1 (PCV1), was isolated from a persistently infected porcine kidney cell line PK-15, and shown to be non-pathogenic to pigs (19, 20). A variant strain of PCV, designated porcine circovirus type 2 (PCV2), was recently isolated from piglets with postweaning multisystemic wasting syndrome (PMWS) in pigs (1, 12). PCV2 is the primary causative agent of PMWS characterized by severe progressive weight loss, dyspnea, lymph node enlargement, diarrhea, pallor, and jaundice in pigs of 7-15 weeks of age (5, 12).

Like PCV1, the genome of pathogenic PCV2 also contains 2 functional ORFs: ORF1 encodes the Rep proteins responsible for virus replication (11), while ORF2 encodes the immunogenic capsid protein (3, 8, 15). Recently, a 3rd ORF, ORF3, was reportedly identified in PCV2 to encode a novel protein and is presumably translated in the anti-sense direction in the ORF1 region (10). It has been reported that the ORF3 of PCV2 cause

apoptosis in PK-15 cells through the activation of caspase 3 and 8 pathways (10). Pathogenesis of an ORF3-deficient mutant PCV2 was evaluated in BALB/c mice (9). The ORF3-deficient mutant was reportedly found to be less pathogenic in mice than the wild-type PCV2 (9). However, the pathogenicity of the ORF3-deficient PCV2 in its natural host, pigs, is not known. The pathogenesis data from a mice study are difficult to interpretate since mice are not the natural host of PCV2 and do not support efficient replication of PCV2.

To definitively identify if ORF3 indeed encodes a novel protein and to assess the role of ORF3, if any, in PCV2 pathogenesis in its natural host, pigs, we characterized ORF3 expression in infected cells and constructed an ORF3-null mutant by mutating the ORF3 start codon from ATG to GTG without changing its coding amino acid in the antisense direction, and conducted a comparative pathogenesis in pigs.

V. Objectives:

(1). To construct an infectious DNA clone of PCV2 with a silenced ORF3 gene. This ORF3-null PCV2 mutant will first be tested *in vitro* to verify its viability and the lack of apoptotic activity.

(2). To compare the pathogenicity of the ORF3-null PCV2 mutant with that of the wild-type pathogenic PCV2 in SPF pigs.

VI. Materials and Methods:

Generation of anti-peptide ORF3-specific rabbit antibody. We initially obtained from Dr. Kwang's group in Singapore an anti-ORF3 Guinea pig antibody that was reported to detect high level of ORF3 protein expression in PCV2-infected cells. Unfortunately, by using this antibody, after repeated attempts we could not detect ORF3 protein in PCV2-infected cells (we were later told that the antibody they sent us was "not correct"). In order to confirm the expression of ORF3 protein, we thus generated n anti-peptide ORF3-specific antibody in rabbit. Briefly, a C-terminal ORF3 peptide (Acetyl-CSRQVTPLSLRSRSSTFNK-COOH) was chemically synthesized at 21rst Century Biochemicals Inc. The peptide was purified, analyzed by HPLC, and conjugated to immune carrier with MBS for immunization of two rabbits. Each rabbits received 5 immunizations, and pre-immune serum and 5 production bleeds were performed. The final anti-ORF3 antibody has a high ELISA titer (>1:10,000).

Construction of an ORF3-null PCV2 mutant. Site-directed mutagenesis was performed on PCV2 infectious DNA clone (isolate 40895) to silence the ORF3 gene using the QuikChange II site-directed mutagenesis kit acoording to the supplied protocol (Stratagene, La Jolla, CA). Primers ORF3mufo (5' GGGATGGTTACCACGGTGAAGAAGTGGTTGTTA 3') and ORF3mure (5' TAACAACCACTTCTTCACCGTGGTAACCATCCC 3') were designed to mutate the start codon of ORF3 from ATG to GTG (M to V) to silence the ORF3 gene. The resulting ORF3-null mutant was sequenced for the

region spanning the ORF3 start codon to verify that the correct mutation was introduced. The muPCV2 is infectious when transfected into PK-15 cells.

***Assessment of the pathogenecity of the muPCV2 in pigs*.** Thirty-one pigs were divided into 3 groups of 11, 10, and 10 each. Prior to inoculation, the pigs were tested negative for PCV2 antibodies. Eleven pigs in group 1 were each inoculated intramuscularly with PBS buffer as negative controls, ten pigs in group 2 were each inoculated intramuscularly with 200 µg of muPCV2 infectious DNA clone (6), and ten pigs in group 3 were each inoculated intramuscularly with 200 µg of PCV2 infectious DNA clone (2). We have previously demonstrated that pigs inoculated intramuscularly with infectious PCV2 DNA clones initiated active PCV2 infection and lesions indistinguishable from pigs inoculated with live PCV2 virus, and thus PCV2 DNA clone can replace live virus for use in pathogenesis studies in pigs (2, 3). Each pig was weighted weekly, and blood was collected from each pig prior to inoculation and weekly thereafter. All pigs were necropsied at 35 days postinoculation (DPI).

***Evaluation of gross and histological pathology*.** Lungs, tonsil, tracheobronchial lymph nodes (TBLN), mesenteric lymph nodes (MSLN), and superficial inguinal lymph nodes (SILN) of all pigs were evaluated for gross pathology during necropsy. Lungs were examined *in situ* for gross lesions. Lymph nodes enlargement was measured for the length, width and total area (cm) of tonsil, TBLN, MSLN, and SILN. For evaluation of histological lesions, sections of lung tissue, tonsil, TBLN, MSLN, SILN, and spleen were collected at necropsy and fixed in 10% neutral-buffered formalin. Microscopic lesions were evaluated in a blinded fashion by a board-certified veterinary pathologist (T.L.). Lung tissue lesions were scored for presence and severity of interstitial pneumonia ranging from 0= normal to 6= severe as previously described (4). Tonsil, spleen, and lymph nodes (tracheobronchial, mesenteric, and superficial inguinal) were scored for the presence of lymphoid depletion ranging from 0= normal to 3= severe lymphoid depletion with loss of lymphoid follicle structure and for the presence of inflammation ranging from 0= normal to 3= severe histiocytic-to-granulomatous inflammation with replacement of follicles (17).

***Detection of PCV2 antigen by immunohistochemistry and PCV2 specific antibodies by ELISA*.** Immunohistochemistry (IHC) was done on formalin-fixed and paraffin-embedded sections of lung tissue, TBLN, SILN, MSLN, spleen, and tonsil for the detection of PCV2-specific antigen as previously described (18). The IHC slides were evaluated and scored in a blinded fashion for the amounts of PCV2 antigen in these tissues. The score ranged from 0= no detectable staining to 3= large numbers of PCV2-antigen diffusely distributed in the tissue section (17). ELISA was performed as previously described to determine serum IgG antibodies to PCV2 (14). A sample/positive (S/P) ratio > 0.2 is considered positive (14).

***Quantitative polymerase chain reaction (Q-PCR) to determine serum viral load*.** All pig sera samples collected prior to inoculation and at different days post-inoculation (DPI) were tested for PCV2 virus DNA load

by Q-PCR as previously described (7). Using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), viral DNA was extracted from 200 µl of a serum sample according to the manufacturer's protocol, and eluted in 100 µl of elution solution. Quantification of viral genomic copies per ml (GC/ml) of serum were then calculated as previously described (3).

VII: Results:

Lack of detection of ORF3 protein expression in PCV2-infected PK-15 cells. By using anti-peptide ORF3-specific rabbit antibody, we could not detect ORF3 protein in PCV2-infected or PCV2 infectious clone-transfected PK-15 cells by IFA or by Western blot analysis. The antibody we received from Dr. Kwang's group also failed to detect ORF3 protein expression in infected cells either. Thus we could not confirm the published reports (9, 10).

Clinical evaluation of pigs. There was no significant difference in pig weights between treatment groups (data not shown) for the duration of the study.

Grossand histological pathology. One pig inoculated with muPCV2 (ID 167) had consolidation in half of the right cranial lung lobe, but other pigs had no visible lung lesions. There were no significant differences in tonsil length ($P = 0.4004$), width ($P = 0.3759$), or total area ($P = 0.6230$) between two treatment groups. No significant differences were found between treatment groups for TBLN and MSLN length ($P = 0.3852$ and $P = 0.1766$, respectively), width ($P = 0.4240$ and $P = 0.9708$, respectively), or total area ($P = 0.6032$ and $P = 0.3885$, respectively). SILN width did not differ significantly between treatment groups ($P = 0.9637$). However, in PCV2-inoculated pigs, the length and total area of SILN were significantly larger than those of PBS-inoculated pigs ($P = 0.0046$ and $P = 0.0137$, respectively). The total area of SILN in muPCV2-inoculated pigs was significantly larger than PBS-inoculated pigs ($P = 0.0179$), although there was no significant difference in the SILN length between muPCV2- and PBS-inoculated groups ($P = 0.0593$). The histological lesion scores and number of lesions present in the tissues were variable between treatment groups (Table 1). Many pigs in both PCV2-inoculated and muPCV2-inoculated groups had interstitial pneumonia, lymphoid depletion in lymph nodes, spleen and tonsil, and histiocytic replacement in lymph nodes. No significant differences in lesion scores were found between treatment groups.

PCV2 antigen and antibody level. Variable levels of PCV2 antigen were detected in lung tissue, TBLN, SILN, MSLN, spleen, and tonsil of muPCV2- and PCV2-inoculated pigs (Table 2). There were no significant differences in the amounts of antigen detected in lung tissue ($P = 0.3620$), TBLN ($P = 0.2282$), tonsil ($P = 0.0634$), spleen ($P = 0.1034$), or SILN ($P = 0.1217$) between treatment groups. Regarding the PCV2 antibody, all pigs were negative for PCV2 antibodies at -8 DPI. None of the PBS-inoculated control pigs

seroconverted to PCV2 over the time course of the study. Beginning at 14 DPI and continuing through the end of the study, there were significant differences in S/P ratios between PCV2 and muPCV2-inoculated pigs ($P < 0.0001$). At 21 DPI, 3 PCV2-inoculated pigs seroconverted to PCV2 antibodies, and by 28 DPI, all PCV2-inoculated pigs were seroconverted. Pigs inoculated with muPCV2 did not seroconvert until 34 DPI, at which point 2 pigs had S/P ratios > 0.2.

Serum viral load. All pigs were negative for PCV2 DNA at -8 DPI. One pig inoculated with PBS (ID 197) had PCV2 DNA detected in serum at 34 DPI (1.14 x 10^6 GC/ml). Since all PBS-inoculated controls were seronegative throughout the study, this sample could be due to lab contamination during the PCR process. All other PBS-inoculated pigs were negative for PCV2 DNA for the duration of the study. PCV2-inoculated pigs developed viremia beginning at 7 DPI, whereas muPCV2-inoculated pigs had a delayed viremia beginning at 21 DPI. There was a significant difference in serum viral DNA load (GC/ml) between PCV2- and muPCV2-inoculated pigs at 14 ($P = 0.0043$), 21 ($P = 0.0015$), and 28 DPI ($P = 0.0018$). Fewer muPCV2-inoculated pigs developed viremia when compared to PCV2-inoculated pigs.

Viruses recovered from sera of PCV2-inoculated pigs 510 and 506 at DPI 28 contain the intact ORF3 start codon and were identical to their PCV2 plasmid infectious DNA clone inoculum. Viruses recovered from sera of muPCV2-inoculated pigs 174, 168, 166, and 175 at DPI 28 has a silenced ORF3 start codon, and were identical to their muPCV2 plasmid infectious DNA clone inoculum.

VIII. Discussion:

PCV2 is the primary causative agent of PMWS, but the molecular mechanism of PCV2 pathogenesis is not known. It is speculated that apoptosis is involved in PCV2 pathogenesis although several groups cannot link apoptosis to PCV2 infection. The recent report on the identification of a 3rd ORF (ORF3) in PCV2 that reportedly induced apoptosis in PK-15 cells has not been independently confirmed (10). An ORF3-deficient PCV2 was showed to be less pathogenic in BALB/c mice when compared to wild-type PCV2 (9). However, whether mouse is a valid model for PCV2 diseases, or even for PCV2 infection, remains highly debatable. Thus, it is important to assess the pathogenecity of the ORF3-deficient PCV2 in its natural host, pigs.

In this study, by using anti-peptide ORF3-specific antibody, we could not detect the expression of ORF3 protein and thus could not confirm the earlier reports (9, 10). We also compared the pathogenecity of an ORF3-null mutant PCV2 (muPCV2) and the wild-type PCV2 in a relevant pig model. We found that the serum viral DNA loads in muPCV2-inoculated group are significantly lower at 14, 21, and 28 DPI ($P < 0.005$) than in wild-type PCV2-inoculated group. However, the results from gross pathology and histopathology do not support the conclusion that the ORF3-deificient PCV2 is less pathogenic as reported by Liu et al (10), as there were no differences between the PCV2-inoculated and muPCV2-inoculated groups in gross lesion score, histological

scores or the number of pigs with interstitial pneumonia, lymphoid depletion in lymph nodes, spleen and tonsil, or histiocytic replacement in lymph nodes.

It has been reported that, when the start codon for the 17 kDa nonstructural protein of the infectious bursal disease virus (IBDV) was silenced, the virus was still able to replicate *in vitro* (13) and *in vivo* (21) but did not cause bursal lesions in chickens (21). The 17 kDa IBDV nonstructural protein was subsequently found to cause apoptosis *in vitro* (22). Another circovirus, chicken anemia virus (CAV), encodes apoptin that causes apoptosis in lymphoblastoid T cells and is involved in pathogenesis (16). However, unlike IBDV and CAV, the reported apoptotic activity of the PCV2 ORF3 (9, 10) has not been independently confirmed, and the reported attenuation of the ORF3-deficient PCV2 in mice (10) is not fully supported by the data obtained from this relevant pig model.

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1 **Table 1. Histological lesions in pigs inoculated with PBS buffer, muPCV2 or PCV2**
2 **infectious DNA clone**

Inoculum	No. of pigs with lesions/no. tested [a]								
		Tracheobronchial Lymph Node		Mesenteric Lymph Node		Superficial Inguinal Lymph Node		Spleen	Tonsil
	Lung	LD	HR	LD	HR	LD	HR	LD	LD
PBS	3/11(0.3)	1/11(0.1)	0/11(0)	4/11(0.4)	0/11(0)	1/11(0.1)	0/11(0)	0/11(0)	0/11(0)
muPCV2	8/10(1.3)	9/10(1.0)	3/10(0.3)	8/10(1.0)	5/10(0.5)	6/9[b](0.8)	2/9[b](0.2)	2/10(0.2)	7/10(0.7)
PCV2	5/10(0.7)	3/8[b](0.6)	2/8[b](0.3)	8/10(1.1)	5/10(0.5)	6/10(0.8)	4/10(0.4)	5/10(0.5)	5/10(0.8)

3
4 [a] Values in parentheses are mean histological scores for interstitial pneumonia, lymphoid
5 depletion (LD) for lymph nodes, spleen and tonsil, and histiocytic replacement (HR) for
6 lymph nodes.
7 [b] A few samples were incorrect tissues and were therefore not scored.
8

Table 2. Detection of PCV2 antigen by PCV2-specific immunohistochemistry in tissues of inoculated and control pigs

Inoculum	No. pigs positive/no. tested (average score):					
	Lung	TBLN	SILN	MSLN	Spleen	Tonsil
PBS	0/11 (0)	0/11 (0)	1/11 (0.2)	0/11 (0)[c]	1/11 (0.1)	1/11 (0.1)
muPCV2	1/10 (0.1)	1/10 (0.1)	1/10 (0.1)	2/10 (0.3)[b]	1/10 (0.1)	3/10 (0.3)
PCV2	0/10 (0)	2/9[a] (0.3)	5/10 (0.6)	7/10 (1.5)[b, c]	4/10 (0.5)	5/10 (0.8)

[a] A tissue sample was missing from the slide.

[b, c] Different superscripts within each column indicate a significant difference in the amount of PCV2 antigen in the tissue between groups.