National reduction in porcine circovirus type 2 prevalence following introduction of vaccination

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21 **Abstract**

22 Porcine circovirus type 2 (PCV2), a small, single-stranded circular DNA virus and the
23 causative agent of porcine circovirus associated disease (PCVAD), was first observed in
24 the mid-1990s in pigs with a post-weaning wasting disease. In 2006 the number of
25 PCVAD cases greatly increased, marking it as an important viral pathogen for the United
26 States (US) swine industry. PCV2 vaccines were introduced to the US in 2006 in response
27 to widespread outbreaks of PCVAD. These vaccines were effective in preventing disease,
28 but did not eliminate virus from the animals. In 2006, prior to vaccine use, a study of
29 PCV2 prevalence in pig herds across the US was performed in conjunction with the US
30 National Animal Health Monitoring System. In 2012, 6 years after widespread PCV2
31 vaccination, this study was repeated. Since the introduction of PCV2 vaccines in 2006,
32 viral presence and viral loads have greatly decreased, and a genotypic shift dominated by
33 PCV2b has occurred. Antibody levels have decreased in the pig population, but
34 approximately 95% of sites continue to be antibody-positive. Widespread vaccination has
35 controlled PCVAD and decreased PCV2 prevalence to the point that viremia is not
36 detected on many sites. Thus, continued vaccination may lead to PCV2 elimination in the
37 national herd over time.

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39 Keywords: porcine circovirus 2; swine; PCV2; vaccination, veterinary virology,
40 epidemiology, immunology

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42 **1. Introduction**

43 Porcine circovirus type 2 (PCV2) is a small, single-stranded circular DNA virus. PCV2 is
44 the causative agent of porcine circovirus associated disease (PCVAD), but requires
45 additional factors for disease to manifest (Allan and Ellis, 2000). PCVAD was first
46 observed in the mid-1990s as a wasting disease in pigs (Allan and Ellis, 2000). In 2006, the
47 number of PCVAD cases appeared to increase, marking PCV2 as an important viral
48 pathogen for the US swine industry (Gillespie et al., 2009; Opriessnig et al., 2007). PCV2
49 vaccines were introduced to the US in 2006, in response to the increasing number of
50 PCVAD cases and its impact on the swine industry. PCV2 vaccination is effective in
51 preventing PCVAD, inducing high levels of anti-PCV2 antibodies and reducing the level
52 of PCV2 in serum, but does not eliminate virus from the animal (Fort et al., 2008;
53 Opriessnig et al., 2008a; Opriessnig et al., 2010).

54 In 2006, prior to vaccine availability, a biologic sampling was performed in pig herds
55 across the US and the prevalence of PCV2 virus and antibodies was examined
56 (Puvanendiran et al., 2011). Here, we re-examined the prevalence of PCV2 virus and
57 immunity in pigs and on sites in the US in 2012 to assess the potential impact of intensive
58 vaccination. We observed substantial decreases in viral load and the frequency of positive
59 results at both the pig and site levels. The PCV2 vaccination program implemented in 2006
60 has controlled PCVAD and appears to have successfully decreased the prevalence of virus
61 in the swine population.

62 **2. Material and Methods**

2.1. Serum samples

Serum samples were obtained from 177 grower/finisher or sow swine operations from 12 states throughout the US in the 2012 National Animal Health Monitoring System (NAHMS) fifth swine health survey (http://www.aphis.usda.gov/animal_health/nahms/swine/). Serum was collected by venipuncture in clot tubes from approximately 30 animals per site for 3,933 grower/finisher swine (20 weeks or older, noted hereafter as finishers or from finisher sites) and 1,772 sows or gilts (noted hereafter as sows or from sow sites, parity 0-5+). Sera were separated, aliquoted, and frozen at -20°C until testing. Subsets of the serum samples were used for the analysis of PCV2-specific antibodies (2,044 finisher, 958 sow) and viremia (1,097 finisher, 407 sow).

2.2. Antigen preparation and ELISA procedure

The full length PCV2 replicase antigen (Rep)(Genbank ID JF290418) was PCR amplified using forward 5'CGCGGATCCATGCCCAGCAAGAAGAATG3' and reverse 5'CCGCTCGAGGTAATTTATTTTCATATGGA3' primers. The PCR product was then purified and cloned into a modified pET24b vector (Novagen, Madison, WI) as described previously for the PCV2 capsid antigen (Cap)(Puvanendiran et al., 2011). The PCV2 replicase and capsid antigens were then prepared as previously described (Johnson et al., 2007; Puvanendiran et al., 2011), except that HisPur cobalt agarose resin was used for purification by affinity chromatography (Thermo Fisher Scientific Inc., Waltham, MA). Detection of antibodies to PCV2 Cap and Rep antigens was performed by indirect ELISA as previously described (Puvanendiran et al., 2011) with the plates coated with 100ng/well

of either the Cap or Rep antigen, test sera diluted 1:50 in phosphate-buffered saline with 0.05% Tween-20 (PBST) containing 5% nonfat dry milk (NFDM), pH 7.4, and horseradish peroxidase (HRP)-labeled goat anti-swine IgG (Bethyl Laboratories, Inc., Montgomery, TX) diluted 1:100,000 in PBST containing 5% NFDM, pH 7.4. Positive results were read as absorbance values >0.24, as determined by background, and positive and negative controls run on each plate.

2.3. DNA extraction, viral copy number, and viral genotype determination

Total DNA was isolated from serum using the QIAamp DNA blood mini kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol, with the addition of 10 g poly dA per 200 l AL buffer, and an elution in 50 l of EB buffer (Dvorak et al., 2013a; Dvorak et al., 2013b). Positive and negative control samples were run with each set of DNA isolations.

PCV2 viral DNA copy number was determined using a SYBR green quantitative PCR assay as previously described (Dvorak et al., 2013a; Dvorak et al., 2013b). The assay does not detect PCV1 DNA.

In cases of ambiguous genotype determination (4.7% of samples) a probe-based quantitative TaqMan PCR was run, with PerfeCTaqPCR FastMix II, Low ROX (Quanta Biosciences, Inc., Gaithersburg, MD) containing 250nM of each primer (forward 5' CAGGGCCAGAATTCAACCT3' and reverse 5' AGGAAAAATGGCATCTTCAAC3'), 200nM of probe (either PCV2a probe 5'/56-FAM/RGAKATTTT/ZEN/GTTGGTCCCCC/3IABkFQ/3' or PCV2b probe 5'/56-FAM/CTCAAACCC/ZEN/CCKCWCTGTGCCC/3IABkFQ/3'), and 5 l of isolated DNA

in a 20 l reaction. Reactions were run in a Stratagene MX3005P machine (Agilent Technologies, Santa Clara, CA) with the following cycling conditions; 95°C for 2 min, followed by 40 cycles of 95° for 15 sec and 58°C for 1 min. A standard curve for PCV2b, PCV2a, and positive and negative controls were present on every plate.

2.3. Statistical Methods

Viral copy numbers, genotypes and prevalence results were described and compared to that of the NAHMS 2006 data and a correlation analysis was used to compare parity to viremia and antibody prevalence using GraphPad Prism software (Version 5.0a, GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. PCV2 viral presence before and after widespread vaccination

A total of 1,097 finisher sera from 73 sites and 407 sow sera from 27 sites (approximately 15 samples each per site) were analyzed for PCV2 viral presence using qPCR. PCV2 viremia was observed in 189 finishers (17.2%) and 36 sows (8.8%) (Table 1), greatly reduced from the 82.6% of viremic finishers in 2006 (Puvanendiran et al., 2011). Of the 73 finisher sites examined, 38 sites (52.1%) were negative for PCV2 in all animals examined and 18 of the 27 sow sites (66.7%) were negative (Table 1), whereas in 2006 only one finisher site (0.5%) was negative (Puvanendiran et al., 2011). The remaining finisher (47.9%) and sow (33.3%) sites contained both PCV2-positive and PCV2-negative animals (Table 1). In 2006 the majority of sites (98.9%) contained at least one positive animal

(Puvanendiran et al., 2011). No sites had all animals test positive for PCV2 in 2012, but 17.3% (32 sites) were all positive in 2006 (Puvanendiran et al., 2011). Examination of viral copy numbers in PCV2-positive animals showed that the great majority of positive animals (96.3% of finishers, 100% of sows) had levels of viremia below 10^6 viral copies/ml (Figure 1A and 1B). There was a 22-fold decrease in median viral levels in finishers as compared to 2006 (Figure 1B)(Puvanendiran et al., 2011). The highest viral titers in 2012 were observed in finishers at 4×10^7 viral copies/ml and only 3 finishing pigs had titers above 10^7 viral copies/ml. This amount was a 50-fold decrease from maximal 2006 viral levels, when viral titers $>10^8$ viral copies/ml were observed in 1.5% of positive animals (Figure 1B)(Puvanendiran et al., 2011). In positive sows, there was no statistical association of parity with the level of viremia (data not shown).

3.2. PCV2 genotype distribution before and after widespread vaccination

The genotypes present in viremic pigs (i.e., above the assay sensitivity of 500 viral copies/ml) were determined using qPCR. PCV2b, in 80.4% of finishers and 75.0% of sows, was the genotype most commonly observed in 2012 (Figure 2A). This was an increase in frequency from 50.7% of PCV2b positive finishers observed in 2006. Also, 2.1% of finishers and 13.9% of sows were co-infected with PCV2a (Figure 2A). At the finisher site level, PCV2b alone was substantially more prevalent (62.9%) than PCV2a alone (8.6%) and both genotypes were present on 28.6% of sites. In 2006 roughly equivalent numbers of sites were infected with each genotype (Figure 2B). On sow sites positive in 2012, 44.4% had PCV2b alone and another 44.4% had both PCV2b and PCV2a present, but only one site had only PCV2a (Figure 2B). Distribution of viral copy number

per genotype showed similar viral levels between the 2a and 2b genotypes with 63.6% and 73.7% of finishers containing $<10^4$ viral copies/ml for each genotype, respectively (Figure 2C). More than 99% of sows were negative or had $<10^4$ viral copies/ml (data not shown). All animals positive for both PCV2a and PCV2b contained low levels of virus ($<1.2 \times 10^4$ viral copies/ml, Figure 2C). In both 2006 and 2012 finishers, the distribution of viral load was roughly equivalent for 2a and 2b genotypes, but the median viral loads decreased from about 10^5 copies/ml in 2006 to about 10^3 copies/ml in 2012 for both genotypes (Figure 2C).

3.3. Anti-PCV2 antibody levels before and after widespread vaccination

Anti-PCV2 serum antibody levels in finishing pigs and sows were determined using both a PCV2 capsid (Cap) ELISA and a PCV2 replicase (Rep) ELISA. Cap ELISA antibodies could result from either natural infection or vaccination, whereas Rep antibodies would only be induced by natural infection since Rep is not present in vaccine formulations. In 2012, Cap antibodies were present in 37.6% of finishing pigs and 55.5% of sows, and Rep antibodies in 19.0% of finishers and 33.7% of sows (Tables 2 and 3). Both Cap and Rep antibodies were present in the same pig in 41.0% of finishers and 62.3% of sows (Tables 2 and 3). Interestingly, 59.0% of finishers and 37.7% of sows were antibody negative for both proteins (Tables 2 and 3). In sows, there was no association between antibody prevalence and parity, suggesting that age did not account for the higher antibody presence against PCV2 antigens in sows or in finishing pigs. At the site level, PCV2 Cap or Rep antibodies were present in one or more animals in 94.9% of finisher sites and 98.5% of sow sites (Table 2 and 3).

A comparison of the percentages of antibody positive animals due to natural infection in 2006 and 2012 (2006 Cap ELISA positive animals versus 2012 Rep ELISA positive animals) showed a marked decrease in antibody presence due to natural infection in 2012 (78.8% positive in 2006, 19.0% positive in 2012)(Table 2)(Puvanendiran et al., 2011), indicating that intensive vaccination has contributed over time to a reduction in the frequency of PCV2 infection in US swine herds.

4. Discussion

PCV2 vaccines were introduced to US swine herds in 2006, enabling producers to control PCVAD. Virus, however, was not eliminated from infected animals even though disease was controlled (Fort et al., 2008; Opriessnig et al., 2008a; Opriessnig et al., 2010). The impact of vaccination on PCV2 viral prevalence in finishing pigs and sows was re-examined in 2012, 6 years after implementation of widespread vaccination. The major impacts of vaccination on viral infection were a dramatic reduction in the number of viremic finishing pigs and a reduction in the viremia levels themselves. It was suggested previously that PCVAD occurs when viral titers reach a high level, usually greater than 10^7 viral copies/ml (Brunborg et al., 2004; Dupont et al., 2009; Olvera et al., 2004; Segales et al., 2005). While this perception is not consistent with high levels of viremia in the absence of disease in sampled finishers in the US in 2006 (Puvanendiran et al., 2011), our findings show that intensive vaccination programs maintained for a period of years in the US corresponded with a dramatic decrease in viral loads. Thus, over time, vaccination to prevent disease may eventually lead to the clearance of PCV2 from swine herds in a region or nationally.

PCV2 viremia was not detected in greater than 50% of sites in 2012, in contrast to less than 1% in 2006. Again, this demonstrates a profound decrease in the overall presence of PCV2 virus. While PCR-negative animals may remain infected in tissues or at levels below test detection thresholds, the relatively low prevalence in 2012 of anti-Rep antibodies, indicative of natural infection, supports the interpretation of PCR results that PCV2 elimination is occurring. PCV2 continues to circulate in swine sites, but at low levels, thus reducing the likelihood of transmission both vertically and horizontally. In 2012, PCV2b was the dominant genotype. Interestingly, a global shift from PCV2a to PCV2b as the predominant strain in the field occurred around 2003 (Dupont et al., 2008; Patterson and Opriessnig, 2010). Similarly, PCV2b also was more prevalent in 2011 in the US in vaccinated, non-vaccinated, and PCVAD-affected pigs (Shen et al., 2012). It was suggested that the genotype switch may be due to a PCV2b viral growth advantage, allowing it to outcompete PCV2a in the host (Dvorak et al., 2013b). However, variation in infectivity and virulence between strains as opposed to between genotypes has been observed (Opriessnig et al., 2008b). Here, even though the majority of finishing pigs were infected with PCV2b, the distributions of PCV2a and PCV2b viremia levels were similar, peaking at 10^3 - 10^4 copies/ml (Figure 2C), suggesting that vaccination does not confer a growth advantage to PCV2b. In 2012, viral loads were reduced equally in both genotypes as compared to viremia levels in 2006. This observation indicates that vaccination controls infection by either of the two genotypes, and that the genotypes are equivalent immunologically while different genetically.

PCV2 infection induces antibody responses to Cap and Rep, whereas vaccination induces anti-Cap antibodies alone since the vaccine products are subunit Cap or killed virus, which lacks significant amounts of nonstructural proteins (Kekarainen et al., 2010; Opriessnig et al., 2007). Hence, natural infection may be sensed by ELISA tests responsive to Cap and Rep, whereas vaccine exposure would be responsive to Cap alone. Infection and vaccination combined would also produce positive results to both Cap and Rep. The high levels of anti-Cap positivity on sites (90.5% and 97.1% on finisher and sow sites, respectively, Tables 2 and 3) and anti-Rep positivity (73.7% and 94.1% on finisher and sow sites, respectively, Tables 2 and 3) is consistent with maintenance of PCV2 infection in sow herds, but a reduction in infection status of finisher herds. Even though PCV2 appears to be maintained in herds, especially sow herds, the frequencies of infected finisher sites and individuals (47.9% and 17.2%, respectively, Table 1) in 2012 is substantially reduced compared to 2006 pre-vaccination frequencies of 99.5% and 82.6% for sites and individuals, respectively (Puvanendiran et al., 2011). Reductions in viremic infections paralleled the serological findings. In finishing pigs, both the number of Rep-antibody positive animals and viremic animals were low (19.0% and 17.2%, Tables 1 and 2). In sows, the number of Rep antibody positive animals was higher than in finishers and the number of viremic animals was lower (33.7% and 8.8%, Tables 1 and 3). By contrast, in 2006, the percent of antibody positive and viremic animals were both over 75% (78.8% and 82.6%, Puvanendiran et al., 2011). Taken together, it appears that vaccination has reduced viral loads in US swine, and that monitoring antibody

responses to Rep and Cap, in combination with PCR monitoring of viremia, is an effective approach to assessing infection and immune status.

Vaccine-based protection against PCVAD has been observed in the absence of detectable antibody responses, suggesting that antibodies are not necessary and that cell-mediated immunity also plays a role in protection against PCV2 infection (Fenaux et al., 2004; Fort et al., 2009; Pérez-Martín et al., 2010). The low prevalence of Rep antibodies in animals in 2012 suggests that piglets are not infected at birth, or maternal antibodies are controlling vertical infection to such an extent that the piglet immune system does not receive a sufficient PCV2 stimulus. Perhaps Cap antibodies and cell-mediated immunity induced by vaccination are indeed able to control natural infection to the extent that the piglet immune system does not induce detectable levels of Rep antibodies that carry into finishing age. Since the introduction of PCV2 vaccines in 2006, viral presence and viral loads have decreased and PCVAD has been controlled in the swine population. Pigs today have lower viral loads, and virus is not detected by PCR in the majority of sites suggesting that continued vaccination has led to PCV2 negative herds over time. While the mechanism by which vaccination controls infection has not been resolved, continued vaccination appears to be driving a dramatic reduction in PCV2 prevalence in swine.

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Figure Legends

Figure 1 – PCV2 viral levels before and after widespread vaccination. PCV2 DNA was
isolated from pig serum and viral copy number was determined by qPCR using a standard
curve. (A) The distribution of viremia levels in finishing pigs from 2012. Negative is <500
copies/ml. (B) Viral copy number distributions in PCV2 positive finishing pigs or sows in
2006 and 2012. Data are shown as box and whisker plots with the whiskers representing

the minimum and maximum values. The cut-off for PCV2 positive samples was 500 viral copies/ml of serum.

Figure 2 – PCV2 genotype distribution and viral levels before and after widespread vaccination. Viral presence and copy number in finishing pig serum from 2006 and 2012 and sow serum in 2012 were compared by genotype. The percent of positive finishing pigs and sows (A) or sites (B) distributed by genotype was determined by qPCR. (C) The percent of positive finishers in 2006 and 2012 distributed by genotype and copy number is shown. Viral copy numbers were determined by a standard curve. The cut-off for PCV2 positive samples was 500 viral copies/ml of serum.

Table 1. PCV2 viral presence in 2012 at the sample and site levels.

	FINISHERS				SOWS			
	Per Animal Presence		Per Site Presence		Per Animal Presence		Per Site Presence	
	N	%	N	%	N	%	N	%
Positive	189	17.2	35	47.9	36	8.8	9	33.3
Negative	908	82.8	38	52.1	371	91.2	18	66.7
Total	1097	100	73	100	407	100	27	100

Table 2. PCV2 Cap and Rep antibody presence in finishers in 2012 at the sample and site level.

	CAP ANTIBODIES				REP ANTIBODIES				CAP or REP ANTIBODIES			
	Per Animal Presence		Per Site Presence		Per Animal Presence		Per Site Presence		Per Animal Presence		Per Site Presence	
	N	%	N	%	N	%	N	%	N	%	N	%
Positive	769	37.6	124	90.5	389	19.0	101	73.7	839	41.0	130	94.9
Negative	1275	62.4	13	9.5	1655	81.0	36	26.3	1205	59.0	7	5.1
Total	2044	100	137	100	2044	100	137	100	2044	100	137	100

Table 3. PCV2 Cap and Rep antibody levels in sows in 2012 at the sample and site level.

	CAP ANTIBODIES				REP ANTIBODIES				CAP or REP ANTIBODIES			
	Per Animal Presence		Per Site Presence		Per Animal Presence		Per Site Presence		Per Animal Presence		Per Site Presence	
	N	%	N	%	N	%	N	%	N	%	N	%
Positive	532	55.5	66	97.1	323	33.7	64	94.1	597	62.3	67	98.5
Negative	426	44.5	2	2.9	635	66.3	4	5.9	361	37.7	1	1.5
Total	958	100	68	100	958	100	68	100	958	100	68	100

A Figure 1

B