

## Dynamics of porcine circovirus type 2 infection and neutralizing antibodies in subclinically infected gilts, and the effect on their litters<sup>□</sup>

*Dinámica de la infección por el circovirus porcino tipo 2 y títulos de anticuerpos neutralizantes en las cerdas de reemplazo subclínicamente infectadas, y el efecto en sus camadas*

*Dinâmica da infecção pelo circovirus porcino tipo 2 e títulos de anticorpos neutralizantes em porcas nulíparas subclínicamente infectadas e efeito em sua leitegada*

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### Summary

**Background:** porcine circovirus type 2 (PCV2) is associated with reproductive disease in newly populated herds and in replacement breeding stock from new sources and is almost exclusively reported in gilts. **Objective:** the main purpose of this study was to assess the dynamics of porcine circovirus type 2 infection and neutralizing antibodies in subclinically infected gilts and the effect on their piglets. **Methods:** the study was conducted with 40 gilts selected at random from four breeding herds. Blood samples, nasal and vaginal swabs were obtained from the gilts at arrival, acclimatization, farrowing, and one day after farrowing. Colostrum samples were collected immediately after parturition and one day after farrowing. Blood, nasal swab, or tissue samples were collected from four piglets prior to suckling. All serums were analyzed by virus neutralization test (VNT) to establish the presence of antibodies. All samples were subjected to SYBER Green real-time PCR assay to detect PCV2 DNA. **Results:** high levels of viremia and viral load of PCV2 in nasal and vaginal swabs were found in healthy gilts at arriving, confirming the introduction of infected animals into the farms. In addition, most gilts were positive for PCV2 DNA in serum, nasal and vaginal swabs at farrowing. PCV2 shedding was also observed in nasal and vaginal fluids and colostrum even in presence of serum neutralizing

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antibodies (NA). Subclinically infected dams had detectable viremia, developed anti-PCV2 antibodies, and there was PCV2 DNA in tissue samples of their born alive and healthy piglets. PCV2a and PCV2b genotypes were confirmed in PCV2 subclinical infection in both dams and piglets *in utero*. **Conclusion:** replacement gilts can be infected with PCV2 before entering the farm and continuous exposure seems to occur horizontally in acclimatization and gestation units or before farrowing. Exposure and infection during gestation may result in infected but apparently healthy piglets.

**Keywords:** gilts, piglets, porcine circovirus type 2, SYBR Green real-time PCR.

## Resumen

**Antecedentes:** el circovirus porcino tipo 2 (PCV2) es asociado con casos de falla reproductivas en granjas recién pobladas, en granjas de cría para cerdas jóvenes y casi exclusivamente en cerdas de reemplazo. Los signos clínicos descritos son: incremento en los abortos durante la segunda y tercera etapa de la gestación, fetos momificados, mortinatos y el nacimiento de lechones débiles no viables. **Objetivo:** el principal propósito de este estudio fue evaluar la dinámica de la infección por el circovirus porcino tipo 2 y títulos de anticuerpos neutralizantes en las cerdas de reemplazo subclínicamente infectadas y el efecto en su camada. **Métodos:** este estudio se realizó con 40 cerdas de reemplazo seleccionadas al azar en cuatro granjas porcinas de cría. De cada animal se colectaron muestras de sangre, hisopados nasales y vaginales al ingresar a la explotación, durante la cuarentena, en el momento del parto y un día post-parto. Igualmente, se colectaron muestras de calostro al terminar el parto y un día post-parto. De cuatro lechones neonatos, se colectaron muestras de sangre, hisopado nasal y tejidos antes de consumir calostro. Todos los sueros fueron analizados mediante la técnica de sero-neutralización para detectar anticuerpos anti-PCV2 y todas las muestras se analizaron por una técnica SYBER Green en tiempo real para detectar el ADN viral. **Resultados:** la detección de un alto nivel de viremia y la demostración de la eliminación viral en hisopados nasales y vaginales permitió demostrar la introducción a las granjas de cerdas de reemplazo infectadas, aparentemente sanas. Igualmente, el suero y los hisopados nasales y vaginales fueron positivos por PCR SYBER Green en la mayoría de las hembras al parto. Se demostró eliminación viral en fluidos nasales, vaginales y en calostro en presencia de anticuerpos séricos neutralizantes. La infección de las cerdas se manifestó en viremia, en el desarrollo de anticuerpos frente al PCV2 y en la presencia del ADN viral en los tejidos de lechones neonatos aparentemente sanos. Los genotipos PCV2a y PCV2b fueron detectados en la infección *in utero*. **Conclusiones:** las cerdas de reemplazo pueden estar infectadas con el PCV2 antes de ingresar a las explotaciones de cría o pueden infectarse por transmisión horizontal durante la cuarentena y gestación. La exposición e infección viral de las cerdas durante la gestación puede resultar en infección subclínica de los lechones neonatos.

**Palabras claves:** cerdas, circovirus porcino tipo 2, lechones, SYBR Green PCR en tiempo real.

## Resumo

**Antecedentes:** o circovírus suíno tipo 2 (PCV2) está associado a casos de falha reprodutiva em granjas recém-assentadas e fazendas de criação de marrãs e afeta principalmente a porcas nulíparas. Clinicamente observam-se aumento dos fetos abortados no segundo e terceiro estágios da gravidez, fetos mumificados, natimortos e nascimento de leitões inviáveis. **Objetivo:** o principal objetivo deste estudo foi avaliar a dinâmica da infecção pelo circovirus porcino tipo 2 e os títulos de anticorpos neutralizantes em porcas nulíparas com infecção subclínica e o efeito em sua leitegada. **Métodos:** este estudo foi realizado com 40 porcas em quatro granjas selecionadas aleatoriamente. Foram coletados de cada animal amostras de sangue, esfregaços nasais e vaginais ao entrar na fazenda, durante a quarentena, no parto e um dia pós-parto. Também foram coletadas amostras de colostro no parto e um dia pós-parto. Amostras de sangue, esfregaços nasal e tecido foram tomadas de quatro leitões antes de consumir colostro. As amostras foram analisadas pelo PCR Sybr Green para detectar e quantificar o PCV2. A detecção de anticorpos contra o vírus do PCV2 em soro foi realizada pelo teste de soroneutralização e todas as amostras foram analisadas através da técnica de SYBR Green PCR para a detecção do ADN viral. **Resultados:** a detecção de um nível elevado de viremia e a demonstração da excreção viral em esfregaços nasais e vaginais nas fêmeas permitiram demonstrar a introdução de porcas nulíparas aparentemente saudáveis. Igualmente, o soro e as secreções vaginais e nasais foram positivos por PCR SYBR Green em tempo real na maioria das porcas no parto. Observou-se excreção viral em secreções vaginais e nasais e colostro na presença de anticorpos neutralizantes. A infecção das porcas manifestou-se no desenvolvimento de anticorpos neutralizantes e detecção de infecção fetal em leitões recém-nascidos aparentemente saudáveis, confirmando a transmissão vertical do PCV2. Os genótipos PCV2a e PCV2b foram

detectados na infecção *in utero*. **Conclusões:** as porcas nulíparas podem estar infectadas com PCV2 antes de entrar nas granjas de criação e pode ser infectadas por transmissão horizontal durante a quarentena e gravidez. Exposição e infecção viral durante a gestação pode resultar em infecção subclínica de leitões recém-nascidos.

**Palavras chave:** *circovirus suíno 2, leitões, porca, SYBR Green PCR em tempo real.*

## Introduction

Porcine circovirus type 2 (PCV2), a member of the *Circoviridae* family, is distributed worldwide and is considered an important emerging pathogen associated with several different syndromes and diseases in pigs, collectively grouped as porcine circovirus-associated diseases (PCVAD; Chae, 2005). PCV2-associated systemic infection is clinically characterized by wasting, dyspnea, and lymphadenopathy and might be associated with diarrhea, pallor, and jaundice (Harding, 2004). The most relevant histological lesions in this condition occur in lymphoid organs and consist of extensive lymphocytic depletion, macrophage infiltration, a few multinucleated giant cells, and botryoid basophilic cytoplasmic inclusion bodies (Rosell et al., 1999).

Reproductive losses attributed to PCV2 infection are less commonly reported. The reproductive failure associated with PCV2 was first described in Canada in 1999 (West et al., 1999) as the causative agent of abortion in a single litter of a herd experiencing late-term abortions as well as increased incidence of stillborn and mummified piglets in a new farm that had been stocked with un-bred gilts. In mature breeding animals, PCV2-associated reproductive failure can manifest as abortion, but it is more frequently associated with increased rates of mummified, macerated, stillborn and weak-born piglets (West et al., 1999; O'Connor et al., 2001). The experimental intrauterine infection with PCV2 resulted in virus replication in the fetuses (Sánchez et al., 2001; Madson et al., 2009a; Saha et al., 2010), reproductive disease associated with fetal pathology (Johnson et al., 2002) and early embryonic death during intrauterine PCV2 infection (Mateusen et al., 2007). Other case reports of reproductive failure implicated PCV2 either as the sole agent or in conjunction with other reproductive disease agents (Brunborg et al., 2007; Castro et al., 2012).

Gilts are fundamental to farm productivity and their introduction represents one of the most critical factors

for the sanitary status of the farm. Taking into account that PCV2-associated reproductive failure outbreaks are typically reported in gilt-start-ups or new farms and it is related to seronegative populations (West et al., 1999; Josephson and Charbonneau, 2001; O'Connor et al., 2001; Pittman, 2008) it is necessary to understand the role of gilts in PCV2 introduction or transmission to the herd and PCV2 infection of their piglets by vertical transmission. The first PCVAD outbreak in Colombia was reported by Clavijo (2007), with both PCV2a and PCV2b genotypes detected (Rincón, 2013). Since then, the diagnosis has focused on PCV2 systemic disease. However, it is unclear what is the role of gilts in PCV2 transmission occurring under field conditions, especially in farms subclinically infected with PCV2. The main objective of this study was to evaluate the dynamics of porcine circovirus type 2 infection and neutralizing antibodies in gilts subclinically infected and to determine the effect on their litters.

## Materials and methods

### *Ethical considerations*

The Ethical and Animal Welfare Committee of Universidad Nacional de Colombia approved the protocol. The euthanasia procedure followed methods established by the OIE in the Terrestrial Animal Health Code, Chapter 7.6.

### *Farm selection*

The study included four commercial breeding herds that volunteered to participate. The final number of participating farms was based on-farm availability to fulfill the sample criteria requirements (strict supervision of farrowing and sufficient technical skills of farm workers to collect samples from sows and piglets). One of the farms was located in Cundinamarca (Farm A) and the remaining three farms were located in the North-west of Colombia (Farms B, C, and D). Farms B and C were multiplier units, with approximately 1,000

sows, producing replacement gilts. Farms A and B were commercial breeding herds.

At the time of sample collection none of the herds had reported PCVAD-associated problems and all herds were free from porcine reproductive and respiratory syndrome (PRRS) virus, classical swine fever (CSF) and Aujeszky's disease (AD), as determined by periodically performed serological testing on sows of mixed parities or wean-to-finish pigs. Piglets in Farm A were vaccinated against CSF according to Colombia's national eradication program at 60 days of age. In the other farms, located in a CSF free zone, no vaccination was conducted. The exception was Farm D, where vaccinated animals received a single 1 mL dose of Ingelvac® CircoFLEX™ (Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany) intramuscularly administered (left neck muscle). None of the other herds used PCV2 vaccination in the breeding animals. Vaccination against PRRS, AD and swine influenza viruses is not authorized in Colombia. All gilts selected for the study were vaccinated against parvovirus (PPV), *Leptospira* spp. and *Erysipelothrix rhusiopathiae* prior to breeding, following a particular protocol for each herd. Characteristics of the herds are summarized in Table 1.

### Sample collection

Farm managers received a checklist with precise instructions on selection of sows and piglets, sample collection, and sample storage. Regardless of breed, a total of 10 clinically healthy gilts from each farm were randomly selected from a group that was introduced to the acclimatization facilities on the same day. In farm D, the gilts arrived with 30 days of age in contrast to farms A, B, and C where they arrived with 135 to 155 days of age. Blood samples as well as nasal and vaginal swabs were collected from gilts at arrival, acclimatization, farrowing and one day after farrowing in order to establish their sanitary status. Farrowing was supervised, and serum and colostrum samples were collected immediately after parturition and one day post-partum. Four normal looking piglets were arbitrarily selected from each litter, regardless of their weight. The piglets were put apart into a dry box to make sure they had no access to colostrum prior to blood and nasal swab collection. Two piglets from each sow were euthanized on the first day of age and necropsied. Heart, lungs, liver, spleen, lymph node (pool), brain, kidneys, serum, and nasal swab samples were collected from each piglet.

**Table 1.** Farm information.

Attribute	Farm A	Farm B	Farm C	Farm D
Province	Cundinamarca	Antioquia	Antioquia	Risaralda
Size	100 sows	1035 sows	1263 sows	600 sows
Farm type	Farrow-to-wean	Multiplier herd	Multiplier herd	Farrow to finish
Genetic	G&P	YxL	LxLW	Topigs 40
Replacement rate (%)	40	50	48	40
Arrival age (days)	135	155	150	30
Gilts isolation (days)	60	30	30	180
Age first AI	210	210	210	240
PCV2 status	Positive	Positive	Positive	Positive
PRRS status	Negative	Negative	Negative	Negative
Aujeszky status	Negative	Negative	Negative	Negative
PCV2 gilts vaccination	No	No	No	Yes <sup>a</sup>
# sows sampled	10	10	10	10
# piglets sampled	40	40	40	40

<sup>a</sup>Gilts were vaccinated 3-4 weeks age with a one-dose product.

Samples were transported to the laboratory following the WHO Guidance on regulations for Transportation of Infectious Substances 2011 - 2012 (UN 2900). Packaging complied with: (a) a leak-proof primary receptacle (plastic bags for tissues; plastic tubes for serum, colostrum and swabs), (b) a leak-proof secondary packaging with ice pads, and (c) a rigid outer packaging (boxes). Each sample was assigned a unique identification number and sent via courier mail to the laboratory. Upon arrival to the laboratory, the blood was centrifuged, and all tissue, colostrum, swabs and serum samples were stored at  $-70^{\circ}\text{C}$  until testing. All sows were visually monitored weekly for clinical signs of PCV2-associated disease, including weight loss, diarrhea, and dyspnea. All sows were allowed to farrow naturally.

#### *Serum neutralization assay*

Sow and piglet pre-suckle serum samples were tested for presence of anti-PCV2 antibodies using a VNT as described previously (Fort *et al.*, 2007). The procedure was performed with the standard positive and negative serum controls (Rincón, 2014) and mock-infected cell control group. A Colombian field isolate of PCV2b strain CO3709 (Rincón, 2013), with titer of  $10^{6.7}$  TCID<sub>50</sub> was used and the test was optimized by using 200 TCID<sub>50</sub>/well. Once a plate was validated, the VNT<sub>50</sub> in the assay was calculated as the reciprocal of the highest dilution of the serum that was able to block PCV2-infection in PK-15 cells by 50%.

#### *PCV2 detection and quantification and PCV2a/b DNA differentiation*

**DNA extraction.** DNA was extracted from 200 microlitres ( $\mu\text{L}$ ) of serum or 20 mg of tissue homogenate using a commercial kit (QIAamp DNA Mini Kit, Qiagen, USA) according to the manufacturer's instructions. Five mL of colostrum samples were centrifuged at 900 g for 10 min. The upper fat layer was removed and the middle aqueous layer (milk whey) and pellets were collected separately. DNA was extracted from the aqueous layer of the milk using the same commercial kit. The swab specimens were suspended in 1000  $\mu\text{L}$  of sterile PBS solution and vigorously vortexed. The DNA was extracted from 200  $\mu\text{L}$  of the swab PBS solution using the same DNA extraction kit. To test for DNA

extraction contamination, a negative DNA extraction control was included as substrate in each group of processed samples by using DNase/RNase-Free Distilled Water (Invitrogen 10977). The resulting DNA was eluted in 50  $\mu\text{L}$  of DNase/RNase-Free Distilled Water and stored at  $-70^{\circ}\text{C}$  until testing.

**Determination of viral copy number.** Viral DNA copy number was determined by a quantitative SYBR Green real-time PCR using the primers previously described (Dvorak *et al.*, 2013). The SYBR® Green I Master (Roche Diagnostics) containing 1  $\mu\text{M}$  primers (forward 5'-GCCAGAATTCAACCTTMACYTTYC-3' and reverse 5' GCGGTGGAATGMTGAGATT 3') and the plasmid PCV2 DNA kindly donated from Dr. Carl Gagnon (Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec, Canada) were used in the assay. A standard curve was generated for each assay using serially diluted plasmid standards containing  $10^{-1}$  -  $10^{-11}$  copies/ $\mu\text{L}$ . In each run, positive PCV2a DNA of strain PCV2a (CO3009) and PCV2b (CO3739) of sequenced Colombian field types (Rincon, 2014) were also included along with the unknown samples. Specificity of primer sequences was confirmed by comparison of 23 Colombian PCV2 sequences and information from the GenBank DNA database using BLASTn (Altschul *et al.*, 1997). The primers amplified a 118 bp region of the cap gene (ORF2) of PCV2 corresponded to nucleotides 1417 to 1534 as reported.

The amplification was carried out in a volume of 20  $\mu\text{L}$  reaction containing 2  $\mu\text{L}$  of 1  $\mu\text{M}$  of each forward and reverse primers, 10  $\mu\text{L}$  of SYBR® Green I Master x 2 conc. (Roche Diagnostics, Indianapolis, USA), 3 L of PCR-grade water and 5  $\mu\text{L}$  DNA template. The PCR conditions were modified for our purposes as follows: 95  $^{\circ}\text{C}$  for 1 min, followed by 40 cycles of amplification at 95  $^{\circ}\text{C}$  for 1 min, 61  $^{\circ}\text{C}$  for 25 s and 72  $^{\circ}\text{C}$  for 5 s. Melting curve analysis was performed at 95  $^{\circ}\text{C}$  for 1 min, 68  $^{\circ}\text{C}$  for 1 min and 90  $^{\circ}\text{C}$  for 0 s; and cooling at 40  $^{\circ}\text{C}$  for 30 s. All real-time PCR reactions (unknown samples, and controls) were performed by duplicate in neighboring wells on the sample plate. Results are reported as an average of the duplicates. Real-time PCR reactions were run using the LightCycler 480 SYBR® Green I Master (Roche Diagnostics). The Light Cycler software (Roche Diagnostics) for PCR data analysis was used

to determine the characteristic melting temperature ( $T_m$ ) of the PCR products obtained from standard PCV2a and PCV2b used.

*Real-time PCR performance.* The linear portion of the standard curve was found to span  $5.45 \times 10^{-9}$  to  $5.45 \times 10^{-1}$ ; meaning a lower detection limit (or cutoff) of  $5.4 \times 10^{-1}$  copies per 20  $\mu$ L reaction. This cutoff corresponded to a threshold cycle ( $C_t$ ) of 34.11 that was applied to tested samples. The dilution factor was taken into consideration when calculating copies of PCV2 in the original sample. The viral concentration was expressed as the mean  $\log_{10}$  viral DNA copy numbers by mL of serum or swab sample. Tissue quantifications were also expressed by mg of tissue sample. The results of the melting curve analysis from the genotyping of field samples showed that the melting temperatures ( $T_m$ ) of PCV2a and PCV2b PCR products were  $78.74 \pm 0.06$  °C and  $80.66 \pm 0.11$  °C, respectively. A difference between the two melting temperatures of about 1.92 °C was evident and this difference was a reliable method for the discrimination of PCV2a from PCV2b.

#### Statistical analysis

The data obtained from SYBER Green real-time PCR and the titers in the VNT assay for sows and piglets were analyzed by ANOVA (analysis of variance) using the General Linear Model Procedures

of SAS 2000. Comparisons of means were conducted using Duncan's multiple range test. Significance was indicated by a probability of  $p < 0.05$

## Results

### Neutralizing antibodies in sows and piglets

PCV2 neutralizing antibodies were identified in all groups at their arrival. With the exception of farm D, VN titers in sows were similar at arrival, acclimatization, farrowing and post-farrowing. Statistically significant differences ( $p < 0.05$ ) were found for farm D when comparing VN titers at arrival (Figure 1). Regarding antibody titers' distribution within each farm, a high variation was shown, ranging from low titers in few sows (1:16) to very high VN titers ( $\geq 1:4,096$ ). Taking into account the distribution of titers in piglets, positive live-born pre-suckle piglet serum samples were detected in 23.1% of the animals evaluated, with a positive detection rate of 17.5% to 27.5% (Table 2). The distribution of VN titers within each farm ranged from 1:4 to 1:32 and there was no significant difference between VN serum titers (VNT50) between farms.

### Level of PCV2 DNA in sows

Table 3 shows the number of sows with a positive PCV2 SYBER Green real-time PCR result and mean viral DNA copies for each serum, swab sample and

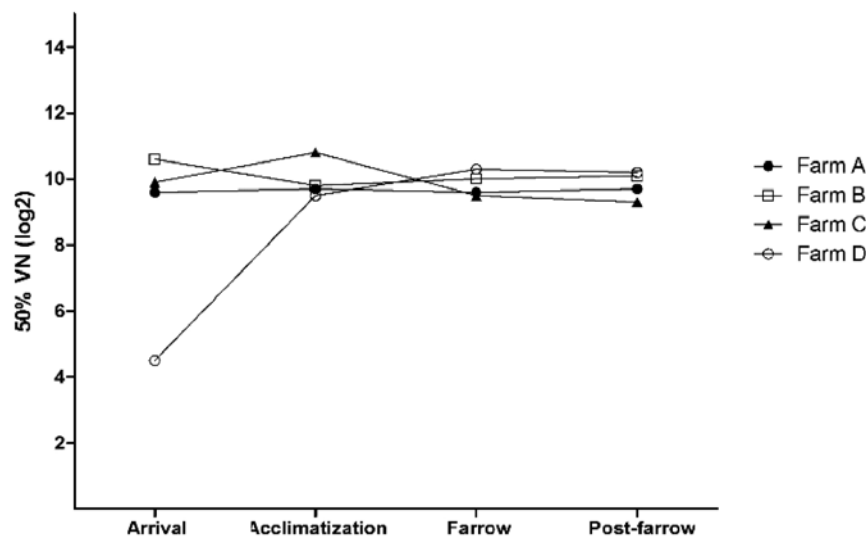


Figure 1. Comparison of the mean VN titers (VNT50) in sows.

**Table 2.** Mean VN titers (VNT50) in the live-born pre-suckle piglet serum samples.

Farm	Sows (No.)	Positive piglets/ total tested	% positive	Mean NA titers (VNT50)*
A	10	11/40	27.5	4.2 <sup>a</sup>
B	10	10/40	25	4.3 <sup>a</sup>
C	10	9/40	22.5	4.1 <sup>a</sup>
D	10	7/40	17.5	4.0 <sup>a</sup>

\*VNT<sub>50</sub> expressed as log<sub>2</sub>.<sup>a</sup>Mean NA titers of piglets with the same letter do not differ significantly (p>0.05).

colostrum for all tested animals in each farm. The analysis of samples from different stages showed differences in the number of PCV2 DNA positive gilts among farms. However, viremic sows were found in each farm at arrival although gilts themselves showed no clinical signs of disease. There was no significant difference between virus level in serum on the four stages for farms A, B, and D. Statistically significant differences (p<0.05) were found for farm C when

comparing viral load in serum samples between acclimatization and farrowing. Nasal and vaginal swabs from sows were examined to determine if the virus was present in secretions, which could then shed into the environment. None of the farms showed statistically significant differences when comparing viral load on nasal swabs among them. However, there was difference on viral load in vaginal swabs collected at arrival and acclimatization as compared with those collected at farrowing in the farm A. In addition, colostrum PCV2 DNA positive samples were detected only in farms B and C and the number of positive colostrum samples within each farm was consistent for both days examined. The viral load was low compared to other fluids tested and there was no significant difference between virus levels in the times studied.

#### *Level of PCV2 DNA in piglets*

Results of virus load in serum, nasal swabs and pool tissues of live-born piglets at farrow and one day post-farrow are presented in Table 4. There was no significant difference between pre-suckle serum virus level in the four farms at farrow. In farm A, tissue viral load was the lowest compared with the other farms.

**Table 3.** PCV2 DNA levels in sows.

	Sample	Farm A	Farm B	Farm C	Farm D
Arrival	serum	5.3*	6.0	5.4	5.4
	nasal swab	6.5	6.1	5.4	4.5
	vaginal swab	6.6 <sup>a</sup>	6.2	4.1	4.7
Acclimatization	serum	5.0	5.9	5.7 <sup>b</sup>	-
	nasal swab	6.8	not attempted	5.2	6.0
	vaginal swab	6.6 <sup>a</sup>	not attempted	5.5	5.2
Farrow	serum	5.0	5.9	4.4 <sup>b</sup>	5.3
	nasal swab	6.1	5.7	5.1	4.8
	vaginal swab	4.5 <sup>a</sup>	5.0	4.7	4.9
Post-farrow	serum	5.8	5.8	4.7	5.9
	nasal swab	5.6	5.8	5.2	5.2
	vaginal swab	4.6	4.9	5.2	5.2

\*Mean log<sub>10</sub> PCV2 DNA copies/mL total DNA per sample in SYBER Green real-time PCR.<sup>a</sup>Significant difference between arrival and acclimatization as compared with farrowing in farm A (p>0.05).<sup>b</sup>Significant difference between acclimatization and farrowing in farm C (p>0.05).

**Table 4.** PCV2 DNA levels in piglets.

	Farm A	Farm B	Farm C	Farm D
Serum	5.1** (30/40)**	5.8 (40/40)	5.8 (4/40)	5.6 (4/40)
Nasal swab	6.0 (1/40)	4.8 (8/40)	5.2 (26/40)	4.2 (19/40)
Tissue pool	2.2 <sup>a</sup> (17/20)	8.1 (1/20)	6.2 (3/20)	4.1 (1/20)

\*Mean log<sub>10</sub> PCV2 DNA copies/mL or mg of total DNA per sample in SYBER Green real-time PCR

\*\*Positive piglets /total tested.

<sup>a</sup>Significant difference between serum and tissue pool from farm A (p>0.05)

### PCV2 genotypes in primiparous sows and piglets

Genotype specific Tm results from SYBER Green real-time PCR in sow samples are shown in Table 5. In the DNA positive PCV2 sows samples, PCV2b was detected at a higher frequency (89.3% for serum, 92.8% for nasal swabs, 96% for vaginal swabs and 100% in colostrum) as compared to PCV2a (10.7% for serum, 7.2% for nasal swabs, 4% for vaginal swabs and 0% in colostrum). *In utero* infection was more common by PCV2b than PCV2a (92.2% for serum, 85.2% for nasal swabs and 100% in tissues). Additionally, PCV2 b was the predominant genotype in all types of analyzed samples regardless of the phase evaluated.

### Discussion

PCV2 was detected in Colombian pigs associated with a wide variety of clinical conditions as described previously (Clavijo, 2007; Rincón, 2013). The data presented here showed that PCV2 infection occurs in gilts from their introduction to breeding herds until their first parity. A high level of viremia and a high viral load of PCV2 in nasal and vaginal swabs detected at the arrival in healthy gilts in this study allows to establish the risk of incorporating subclinically infected animals or new PCV2 virus strains to the breeding herd.

In terms of PCV2 DNA virus load in serum at arrival, there was no relationship between virus level and age; weaning and pre-puberty females showed viremia and furthermore, most gilts were positive for PCV2 DNA in nasal and vaginal swabs, which suggests that they

**Table 5.** SYBER Green real-time PCR results for the detection and genotyping of PCV2 in sows and piglets.

Farm	Sows		Piglets	
	PCV2a	PCV2b	PCV2a	PCV2b
A	3	67	1	47
B	9	62	5	44
C	1	75	1	32
D	6	41	4	20
	19 (7.2%)	245 (92.8%)	11 (7.6%)	143 (92.3%)

came in contact with PCV2 while in the genetic farm. By contrast, very high VN titers were found in farms A, B, and C at arrival (age 135 - 150 days) and low VN titers were detected in farm D gilts at 30 days of age when maternal immunity waned. In addition, it can be speculated that humoral immune response in Farm D sows was not detected after vaccination due to the short time period between vaccination and serum sample collection. Later, the gilts were under constant infection challenge through contact with viremic sows and contaminated environment in the acclimatization, gestation or farrowing units. These observations indicate that PCV2 infection in gilts is persistent before the first parity.

According to the data presented here and field investigations (Opriessnig *et al.*, 2004), it seems that no difference exist between young and adult sows in terms of PCV2 infection because of the widespread presence of PCV2 in the farms. Nevertheless, the introduction of PCV2- seronegative gilts may have been an important factor associated with reproductive inefficiency in the herd that may have predisposed groups of gilts to enter the breeding herd with no active immunity to PCV2. Immunity of replacement gilts to PCV2 should be considered when changing gilt sources and also in low-immunity, high-risk herds, such as all-gilt or low-parity herds, recently populated herds, and herds with high replacement rates (Pittman, 2008).

In the present study, coexistence of NA with viral DNA was observed in gilts subclinically infected at arrival, during the acclimatization period and at farrowing. A possible explanation could be that PCV2 infection prolonged impairment of humoral response.



In a previous study by Meerts *et al.* (2006) it was suggested that post-weaning multisystemic wasting syndrome (PMWS) affecting pigs might be unable to produce antibodies against certain neutralizing epitopes. This hypothesis was based on observations made under experimental and field conditions in which PMWS pigs lacked NA but developed total antibody titers in a similar way to subclinically infected animals. Viral persistence despite high levels of NA was also observed in the experimentally PCV2-infected pigs and has been also reported for chicken anaemia virus, a related virus of the *Circoviridae* family (Brentano *et al.*, 2005). Additionally, it should be noted that pregnant dams were NA seropositive, the sows were viremic at farrowing and PCV2 DNA was shed in sow secretions but they didn't show clinical signs of infection.

In contrast to other experimental and field observations of PCV2-associated reproductive failure (West *et al.*, 1999; O'Connor *et al.*, 2001; Park *et al.*, 2005; Pittman, 2008), abortion, dam illness, or increased numbers of nonviable piglets were not observed under the conditions of this study (data not shown). Differences between studies might be related to differences in virulence of PCV2 strains, management conditions or animal susceptibility. However, the data presented here support that PCV2 infection in gilts may not result in reproductive failure but can be associated with vertical transmission, indirectly demonstrated by PCV2 DNA detection in pre-suckle serum and pool tissues of live-born healthy piglets. In the same way, it was observed that subclinically infected live-born piglets developed NA titers to PCV2 and the mean farm  $\log_{10}$  PCV2 DNA copies/mL of serum ranged from 5.10 to 5.80 in positive animals. The findings in this study confirm and extend previous observations that PCV2 can be vertically transmitted and could be present in large amounts within lymphoid tissues from piglets infected *in utero* (West *et al.*, 1999). PCV2 viremia was detected in 48.7% of live-born healthy piglets, thus confirming that after a maternal viraemic episode there is intra-uterine exposure of fetuses to PCV2 without *in utero* infection in all of them (Sánchez *et al.*, 2001; Pensaert *et al.*, 2004; Yoon *et al.*, 2004; Madson *et al.*, 2009b; Saha *et al.*, 2010; Shen *et al.*, 2010).

Also, results from this study showed coexistence of NA with viral DNA in some of the live-born

healthy piglets suggesting that NA alone might not be enough for viral clearance. In natural cases of PCV2 infection, viral clearance is thought to be mediated by the combination of NA and cell-mediated responses, however the role of NA in fetus needs to be clarified. This way of transmission has been related with the development of multi-systemic disease later in life and pigs infected with PCV2 *in utero* are likely to be more susceptible in the growing phase to co-infections with other pathogens (Ha *et al.*, 2008). Furthermore, the spread of the virus to live-born PCV2-negative piglets might be favored by contact with contaminated environment or horizontal transmission in the farrowing barns.

In this study, infectious PCV2 shedding was observed in nasal and vaginal secretions and in colostrum of unvaccinated first-parity sows even in the presence of high virus load and NA in serum. Previous field reports evaluating infectious PCV2 shedding in colostrum found that 1/33 samples were positive (Shibata *et al.*, 2006). In another study, PCV2 was isolated from 2/6 colostrum samples using experimentally inoculated sows (Ha *et al.*, 2009). In naturally infected sows, infectious PCV2 was detected in 22/41 colostrum samples, 7/20 of vaccinated and in 15/21 of unvaccinated sows (Gerber *et al.*, 2011). Differences in the results between studies may be related to differences in PCV2 infection challenge and virulence, levels of PCV2-antibodies in sows and different test sensitivities. However, the present findings suggest that PCV2 shedding in colostrum could occur at a different frequency than previously reported. Colostrum has a greater number of monocytic cells which might be infected with PCV2 (Park *et al.*, 2009) and although maternal antibodies against PCV2 are present at various levels in the swine population their ability to completely prevent PCV2 infection has not been proven.

Recent studies demonstrated that both genotypes (PCV2a and PCV2b) have been circulating in farrow-to-finish pigs in PCVAD-affected and non-affected Colombian farms (Rincón, 2013). Similarly, this study revealed PCV2 subclinical infection with both genotypes in gilts from commercial and multiplier herds. The results of this research are consistent with previous field and experimental reports, in which both genotypes have been detected in PCV2

infection *in utero* (Lefebvre et al., 2008; Castro et al., 2012). Furthermore, gross lesions of PCV2 fetal infection and high PCV2 titers in tissues were noted across all PCV2 isolates used in experimental studies (Larochelle et al., 2000). In addition, with the information known to date, it appears that genotype differences between PCV2 isolates cannot account for the reproductive manifestations of the virus and no repeatable characteristic amino acid motifs in the PCV2 capsid protein or in the residues used for genotype classification were associated with reproductive failure (Castro et al., 2012).

Due to the combination of vaccine usage and the ubiquitous nature of PCV2, most replacement gilts and sows in breeding herds have been exposed to field PCV2 or PCV2 vaccines prior to breeding. In this study, gilts in farm D were vaccinated against PCV2 between 3 to 4 weeks of age but there was not a significant difference between viral serum load and VN titer in sows in the four farms at farrow and it did not affect viral shedding or fetal PCV2 transmission. It is well known that vaccination of late-gestation PCV2-infected sows results in uniformly high antibody levels at the time of farrowing, but does not affect viral load in sows or PCV2 transmission to piglets (Gerber et al., 2011). Nevertheless, the efficiency of NA response as a result of vaccinating acclimatization sows subclinically infected with PCV2, as well as virus load and *in utero* PCV2 transmission needs to be clarified. It is generally perceived that reproductive failure associated with PCV2 infection is reduced in seropositive dams as it was observed in this study. Nevertheless, further studies are required to determine how different management conditions might affect disease expression associated with PCV2 infection.

In conclusion, gilts can be infected with PCV2 before they are introduced into the farm, and continuous exposure in acclimatization and gestation units appears to occur horizontally. PCV2 subclinically infected dams may shed virus into the environment and also may produce subclinically infected live-born piglets. These findings raise the question of the importance of PCV2 viral infections in the pathology of reproductive failures and reveals that birth of healthy infected piglets may be a common event in conventional and multiplier swine farms. This finding contributes to the understanding of the epidemiology

and transmission of porcine circovirus type 2 (PCV2) in swine populations.

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### Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

### References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; 25:3389-3402.
- Brentano L, Lazzarin S, Bassi SS, Klein TA, Schat KA. Detection of chicken anemia virus in the gonads and in the progeny of broiler breeder hens with high neutralizing antibody titers. *Vet Microbiol* 2005; 105:65-72.
- Brunborg IM, Jonassen CM, Moldal T, Bratberg B, Lium B, Koenen F, Schönheit J. Association of myocarditis with high viral load of porcine type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. *J Vet Diagn Invest* 2007; 19(4):368-75.
- Castro MG, Cruz TF, Salgado VR, Kanashiro TM, Ferrari KL, Araujo JP, Brandão PE, Richtzenhain LJ. Detection of porcine circovirus genotypes 2a and 2b in aborted fetuses from infected swine herds in the State of São Paulo, Brazil. *Acta Vet Scand* 2012; 54:29.
- Chae C. A review of porcine circovirus 2-associated syndromes and diseases. *Vet J* 2005; 169(3):326-336.
- Clavijo JA. Circovirus porcino tipo 2 (PCV2) en Colombia evaluación serológica, caracterización histopatológica de las lesiones y detección del antígeno viral mediante inmunohistoquímica [Tesis Especialista en Anatomopatología Veterinaria]. Universidad Nacional de Colombia. Facultad de Medicina Veterinaria y Zootecnia; 2007.
- Dvorak CM. Cellular pathogenesis of porcine circovirus type 2 infection. *Virus Res* 2013; 174:60-68.
- Fort M, Olvera A, Sibila M, Segales J, Mateu E. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)- affected and non-PMWS-affected pigs. *Vet Microbiol* 2007; 125:244-255.

- Gerber PF, Garrocho FM, Lana AM, Lobato ZI. Serum antibodies and shedding of infectious porcine circovirus 2 into colostrum and milk of vaccinated and unvaccinated naturally infected sows. *Vet J* 2011; 188(2):240-242.
- Ha Y, Ahn KK, Kim B, Cho KD, Lee BH, Oh YS, Kim SH, Chae C. Evidence of shedding of porcine circovirus 2 in milk from experimentally infected sows. *Res Vet Sci* 2009; 86:108-110.
- Ha Y, Lee YH, Ahn KK, Kim B, Chae C. Reproduction of postweaning multisystemic wasting syndrome in pigs by prenatal porcine circovirus 2 infection and postnatal porcine parvovirus infection or immunostimulation. *Vet Pathol* 2008; 45:842-848.
- Harding JC. The clinical expression and emergence of porcine circovirus 2. *Vet Microbiol* 2004; 98:131-135.
- Johnson CS, Joo HS, Direksin K, Yoon KJ, Choi YK. Experimental *in utero* inoculation of late-term swine fetuses with porcine circovirus type 2. *J Vet Diagn Invest* 2002; 14:507-512.
- Josephson G, Charbonneau G. Case report of reproductive problem in a new startup operation. *J Swine Health Prod* 2001; 9:258-259.
- Larochelle R, Bielanski A, Muller P, Magar R. PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. *J Clin Microbiol* 2000; 38:4629-4632.
- Lefebvre D, Barbé F, Atanasova K, Nauwynck H. Inoculation of porcine fetuses with different genotypes and doses of PCV2. In: 20th International Pig Veterinary Society Congress. 2008; 1:38.
- Madson DM, Patterson AR, Ramamoorthy S, Pal N, Meng XJ, Opriessnig T. Effect of porcine circovirus type 2 (PCV2) vaccination of the dam on PCV2 replication *in utero*. *Clin Vaccine Immunol* 2009a; 16(6):830-834.
- Madson DM, Patterson AR, Ramamoorthy S, Pal N, Meng XJ, Opriessnig T. Reproductive failure experimentally induced in sows via artificial insemination with semen spiked with porcine circovirus type 2. *Vet Pathol* 2009b; 46:707-716.
- Mateusen B, Maes DG, Van Soom A, Lefebvre D, Nauwynck HJ. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. *Theriogenology* 2007; 68:896-901.
- Meerts P, Misinzo G, Lefebvre D, Nielsen J, Bøtner A, Kristensen CS, Nauwynck HJ. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Vet Res* 2006; 2:6.
- O'Connor B, Gauvreau H, West K, Bogdan J, Ayrout M, Clark E.G, Konoby C, Allan G, Ellis JA. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite swine production unit. *Can Vet J* 2001; 42:551-553.
- Opriessnig TS, Thacker EL, Halbur PG. Derivation of porcine circovirus type 2-negative pigs from positive breeding herds. *J Swine Health Prod* 2004; 12(4):186-191.
- Park JS, Kim J, Ha Y, Jung K, Choi C, Lim JK, Kim SH, Chae C. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. *J Comp Pathol* 2005; 132:139-144.
- Park JS, Ha Y, Kwon B, Cho KD, Lee BH, Chae C. Detection of porcine circovirus 2 in mammary and other tissues from experimentally infected sows. *J Comp Pathol* 2009; 140:208-211.
- Pensaert MB, Sánchez RE Jr, Ladekjaer-Mikkelsen AS, Allan GM, Nauwynck HJ. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* 2004; 98:175-183.
- Pittman J S. Reproductive failure associated with porcine circovirus type 2 in gilts. *J Swine Health Prod* 2008; 16: 144-148.
- Rincón MA. Circovirosis porcina en Colombia: aspectos moleculares y caracterización biológica de cepas de campo [Tesis Doctorado en Ciencias de la Salud Animal]. Universidad Nacional de Colombia. Facultad de Medicina Veterinaria y Zootecnia, 2013.
- Rosell C, Segales J, Plana-Duran J, Balasch M, Rodriguez-Arriola GM, Kennedy S, Allan GM, McNeilly F, Latimer KS, Domingo M. Pathological, immunohistochemical, and *in-situ* hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *J Comp Pathol* 1999; 120:59-78.
- Saha D, Lefebvre DJ, Van Doorselaere J, Atanasova K, Barbé F, Geldhof M, Karniychuk UU, Nauwynck HJ. Pathologic and virologic findings in midgestational porcine after experimental inoculation with PCV2a or PCV2b. *Vet Microbiol* 2010; 145:62-68.
- Sánchez RE, Nauwynck HJ, McNeilly F, Allan GM, Pensaert MB. Porcine circovirus 2 infection in swine fetuses inoculated at different stages of gestation. *Vet Microbiol* 2001; 83:169-176.
- Shen H, Wang C, Madson DM, Opriessnig T. High prevalence of porcine circovirus viremia in newborn piglets in five clinically normal swine breeding herds in North America. *Prev Vet Med* 2010; 97:228-236.
- Shibata J, Okuda Y, Kitajima K, Asai T. Shedding of porcine circovirus into colostrum of sows. *J Vet Med B Infec Dis Vet Public Health* 2006; 53(6):278-280.
- West KH, Bystrom JM, Wojnarowicz C, Shantz N, Jacobson M, Allan G M, Haines D. M, Clark E G, Krakowka S, McNeilly F, Konoby C, Martin K, and Ellis J A. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* 1999; 11:530-532.
- Yoon KJ, Jepsen RJ, Pogranichniy RM, Sorden S, Stammer R, Evans LE. A novel approach to intrauterine virus inoculation of swine using PCV type 2 as a model. *Theriogenology* 2004; 61:1025-1037.