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RESEARCH ARTICLE

PCV2 Infection in Vaccinated Conventional Gilts Inseminated with PCV2b-Spiked Semen

Carlo Bianco[1,*], Giuseppe Sarli[1], Serena Panarese[1], Maria Laura Bacci[1], Giovanna Galeati[1], Michele Dottori[2], Paolo Bonilauri[2], Davide Lelli[3], Giorgio Leotti[4], Thaïs Vila[5], François Joisel[5] and Fabio Ostanello[1]

[1]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano Emilia, Bologna, Italy; [2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER) – Section of Reggio Emilia, Via Pitagora 2, 42100, Reggio Emilia, Italy; [3]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER), Via A. Bianchi 9, 25124, Brescia, Italy; [4]Merial Italia Spa, Strada 6, Palazzo E/5, 20090 Milanofiori, Assago, Milan, Italy; [5]Merial SAS, 29 avenue Tony Garnier, 69007, Lyon, France
*Corresponding author: carlo.bianco3@unibo.it

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ABSTRACT

The present trial investigated the effect of PCV2 vaccination on viremia, virus shedding and viral load in maternal tissues and foetuses of conventional gilts inseminated with PCV2b-spiked semen. Twelve gilts were randomly divided into two groups of six animals each (vaccinated infected, VI; non-vaccinated infected, NVI). Estrus synchronization was followed by artificial insemination (AI) with a single PCV2 negative semen dose supplemented with 0.2 mL of a PCV2b suspension containing $10^{4.4}$ $TCID_{50}$/50 µL (total viral dose: 10^5 $TCID_{50}$). Vaginal, nasal and faecal swabs, and blood samples were collected weekly from two days before artificial insemination till the end of the experimental period (55 days post AI; DPAI) and tested by real-time PCR (qPCR) for PCV2; sera were tested for anti-PCV2 antibodies. During necropsy foetal and maternal tissues were collected for qPCR and histopathology. In each of the VI and NVI groups three out of the six gilts were pregnant at 29 DPAI. The VI group showed a significantly lower proportion of PCR-positive swabs: 24.6% VI *vs* 71.3% NVI. PCV2 clearance was demonstrated by qPCR in lymphoid tissue during the trial in the VI group. Only one foetus was PCV2-positive (in the NVI group) and three amniotic fluids of the NVI group. PCV2 was found in a significantly lower proportion of the placenta of foetuses in the VI group (39%) than the NVI group (77%). The PCV2 vaccine seems to play an active role in reducing virus shedding, tissue viral load and foeto-placental infection.

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INTRODUCTION

Porcine circovirus type 2 (PCV2) infections is a globally important issue in intensive swine farms. PCV2 rarely causes clinical diseases, but when it does, pleomorphic faceted syndromes manifest, often in combination with opportunistic pathogens. The clinical manifestations are known as porcine circovirus diseases (PCVD) or porcine circovirus-associated diseases (PCVAD) in the EU or USA, respectively (Opriessnig *et al.*, 2007; Segalés, 2012).

PCV2 has been linked to stillbirth, mummification, embryonic death, infertility and abortion (SMEDIA) syndrome, and in recent years it has been established that reproductive disorders characterized by acute events affect gilts or low-parity sows and PCV2-free herds. Manifestations affecting reproduction can be divided into clinical and subclinical events. Clinical events include evident SMEDIA-like disorders with delivery of piglets showing macroscopic and microscopic lesions caused by PCV2, while subclinical events include vertical transmission with delivery of PCV2-infected piglets but without lesions or indication of reproductive failure (Madson and Opriessnig, 2011). Vertical transmission of PCV2 has been successfully demonstrated and reproduced in a number of experimental and field trials (Madson *et al*., 2009a; Hansen *et al.*, 2010; Nathues *et al.*, 2011). Horizontal transmission between gestating sows is equally

important in increasing the risk of viremia that causes vertical transmission to foetuses (Gerber *et al.*, 2012).

An important tool to control PCV2 infection is vaccination of sows to avoid clinical manifestations, reduce the frequency and magnitude of viral shedding, and accordingly cut economic losses. Field evidence and specific field trials have demonstrated that vaccination against PCV2 improves the sow's reproductive parameters by increasing the mean number of live born and weaned piglets per sow per year (Pejsak *et al.*, 2012; Maurin-Bernaud *et al*., 2012).

As gilts are at the highest risk of PCV2-induced reproductive disorders (Madson and Opriessnig, 2011), this paper presents the results of an experimental challenge in controlled conditions designed to evaluate the influence of PCV2 vaccination on the dynamics of PCV2 infection in the dam and its vertical transmission to foetuses in conventional gilts inseminated with PCV2b-spiked semen. We evaluated the ability of the vaccination to reduce and limit not only maternal infection and related events (viremia and shedding), but also vertical transmission to foetuses, focusing on the first half of pregnancy (days 0-55).

MATERIALS AND METHODS

Animals: Twelve Large White conventional female piglets were randomly selected from animals on a breeding farm that had a policy of vaccination against porcine parvovirus (PPV), Aujeszky disease virus (ADV) and erysipelas. They were divided into two groups (vaccinated infected, VI and non-vaccinated infected, NVI) of six gilts each (VI-1-6; NVI-1-6) and group VI gilts were vaccinated at the farm with Circovac® (Merial, Lyon, France, batch L357004) according to the datasheet. At about 160 days of life, the gilts (mean live body weight ± SD: 98.5 ± 9.4 Kg) were housed in individual pens of about 6 m^2 in two separate rooms with restricted access (University of Bologna facilities). On a daily basis, the rectal temperature was measured and a physical examination performed. The experimental procedures were approved by the Animal Experimentation Ethical and Scientific Committee - Alma Mater Studiorum, University of Bologna and subsequently approved by the Italian Ministry of Health. The study was carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes (Council Directive 86/609/EEC).

Experimental design: The days of the trial were indicated by the abbreviation DPAI (days post artificial insemination); 0 DPAI is the time of insemination with semen supplemented with PCV2b. The whole *in vivo* trial took 60 days (from -4 to 55 DPAI).

On -4 DPAI a pharmacological treatment started to induce puberty, oestrus and superovulation: 1500 UI/gilt of Folligon™ (Intervet, Boxmeer, The Netherlands) were intramuscularly administered and then, at -2 DPAI, 750 UI/gilt of Corulon™ (Intervet) were given according to Sarli *et al.* (2012). Insemination with PCV2b-spiked semen was performed on 0 DPAI, approximately 40 hours after the Corulon™ administration. All animals were inseminated once with 100 mL PCV2 negative semen supplemented with 0.2 mL of a PCV2b suspension containing $10^{4.4}$ $TCID_{50}$/50 µL (total viral dose: 10^5 $TCID_{50}$). The PCV2b strain was the same used by Sarli *et al.* (2012). Preliminarily, the semen had been tested by PCR to exclude the presence of PCV2 genome (Sarli *et al.*, 2012).

At 29 DPAI, ultrasonographic diagnosis of pregnancy was performed. Gilts that aborted and non- pregnant animals underwent euthanasia after abortion or ultrasonography to assess the presence of possible lesions, while the pregnant animals were pharmacologically euthanized between 49 and 55 DPAI, adopting the following protocol: a) 8 mL of Stresnil® (Janssen Animal Health, Beerse, Belgium) IM.; b) 25 mL of Ketavet100® (Intervet Productions S.r.l., Milan, Italy) IM after 20 min; c) 10 mL of 50 mg/mL Pentothal Sodium® (Intervet Productions S.r.l., Milan, Italy) IV after 20 min; d) 10 mL of Tanax® (Intervet Italia, Latina, Italy) IV.

In vivo sampling: From -2 DPAI to the day of euthanasia, weekly blood samples, nasal, faecal and vaginal swabs were collected to detect PCV2 viral shedding by means of real-time PCR (qPCR). On 14 DPAI blood sampling was not performed to avoid stress during the maternal recognition of pregnancy. Serum antibody titres against PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), ADV and PPV were determined by testing serial dilutions of each serum sample through competitive ELISA assay (Sarli *et al.*, 2012). The anti-PCV2 antibody titration was performed weekly; antibodies against ADV, PPV and PRRSV were titrated on the first (-2 DPAI) and last (*intra-mortem*) sampled serum. Serum progesterone (ng/mL P4) was detected by validated radioimmunoassay with sensitivity 2.2 pg/tube; intra- assay CV 5.7%; inter-assay CV 10.5%; Sarli *et al.*, 2012).

Post mortem sampling: Gilts underwent full post-mortem examination. Samples of lymphoid organs (lymphoid tissue set: tonsils, inguinal and uterine lymph nodes), reproductive tract [reproductive tissue set: ovary (ovarian functional structures - follicles, corpora lutea, corpora albicantia - were counted), oviduct, uterus, cervix, vagina, feto-placental unit] and other organs [small intestine (duodenum, jejunum, ileum), liver, kidney, heart and lung] were collected. Each foetus was weighted and crown-rump length measured to estimate the gestational age according to Kirkwood *et al*. (2012). Sampled tissue sets from each feto-placental unit comprised: amniotic fluid, allantochorion, spleen, heart and liver (these organs were pooled); to avoid cross-contamination between foetuses sterile surgical tools were used.

Real-time PCR and sequencing: Viral DNA was extracted (from swabs, sera, lymphoid and reproductive tissue sets) and quantified via qPCR for PCV2 (Sarli *et al.*, 2012). To evaluate the identity between the PCV2b strain used for infection and the strain detected in tissues, DNA sequencing was carried out on 10 randomly selected qPCR-positive samples of lymphoid and reproductive organs. PCV2 PCR was run in accordance with Ouardani *et al.* (1999). DNA amplification and quality were then

checked on a 1% agarose gel in 1x TAE buffer. The amplicons were purified with a column-based system (Macherey-Nagel), testing both amplified samples and excised DNA fragments from agarose gel after electrophoresis. The samples were then sent to the Eurofins-MWG-Operon's sequencing service (Ebersberg, Germany). All sequences passed the quality control standards. The amplified ORF2 fragment was sequenced and compared both with Genebank reference sequences (PCV2a AF465211; PCV2b HM038022.1) and the PCV2b strain used for infection (PCV2b 6503, IZSLER, Brescia, Italy). The Basic Local Alignment Search Tool (BLAST) was used to identify sequence similarity, choosing a cut-off of 98% similarity among tested genomes.

Histopathology and immunohistochemistry: All sampled tissues were subjected to routine histological examination (H&E). Immunohistochemistry was run only on qPCR-positive tissues of lymphoid and reproductive tissue sets, with $>10^8$ PCV2 genomic copies/mL using a monoclonal antibody (PCV2 Mab F217), as previously described (Sarli *et al*., 2009). To increase the sensitivity, a streptavidin-biotin-peroxidase polymeric complex (SuperPicture kit peroxidase, ZymedR Lab, San Francisco, USA) was used.

Statistical analysis: The Kolmogorov–Smirnov test for goodness of adaptation was used to verify distribution normality. On the basis of the results of this test, Mann-Whitney (M-W) U test was used to compare quantitative data (PCV2 genomic load of tissues, swabs and blood samples, anti PCV2 antibody titre in animals of the two groups). Wilcoxon signed-ranks test for repeated measures was used to analyze the antibodies dynamics for ADV, PPV and PRRSV. The proportion of PCV2-positive samples (blood, reproductive and lymphoid tissue sets, swabs) was analyzed with Chi-square test. Data were analyzed with the SPSS 19.0 (SPSS Inc., Chicago, USA).

RESULTS

In vivo and post mortem: On 0 DPAI all gilts showed immobility reflex and oestrus signs. After artificial insemination no oestrus signs were detected and a rise in serum P4 was evident (-2 DPAI: P4=0.2±0.28 ng/mL; 7 DPAI: P4=21.91±7.06 ng/mL).

Gilts NVI-5, VI-3 and VI-4 aborted at 20, 22 and 24 DPAI, respectively. At 29 DPAI, ultrasound examination proved pregnancy in VI-1, VI-5, VI-6, NVI-3, NVI-4 and NVI-6 gilts. The serum P4 decreased to low values on 21 DPAI in non-pregnant gilts (1.35±1.75 ng/mL), while pregnant animals maintained high levels of serum P4 (18.60±5.28 ng/mL at 21 DPAI). Non-pregnant animals were sacrificed on 34 (VI-2), 23 (VI-3), 25 (VI-4), 33 (NVI-1), 33 (NVI-2) and 22 (NVI-5) days post artificial insemination (mean 29 DPAI). Pregnant gilts were euthanized on 54 (VI-1), 49 (VI-5), 50 (VI-6), 51 (NVI-3), 49 (NVI-4), 55 (NVI-6) days post artificial insemination (mean 53 DPAI).

Three, 15, 9, 10, 16 and 14 foetuses were collected from gilts VI-1, VI-5, VI-6, NVI-3, NVI-4 and NVI-6, respectively. The size of all foetuses was compatible with gestational age and no gross lesion was observed. Ovarian functional structures were consistent with the number of foetuses (mean±SD corpora lutea: 15±6.6 and 2.6±4.4 in pregnant and non-pregnant gilts, respectively; mean corpora albicantia of non-pregnant animals: 6.4±3.5). The uterus of non-pregnant gilts showed a prepubertal appearance, with a mild ovarian response to pharmacological treatment, and ovarian functional structures were manifestation of a second cycle after the first, induced estrus, not followed by a successful pregnancy.

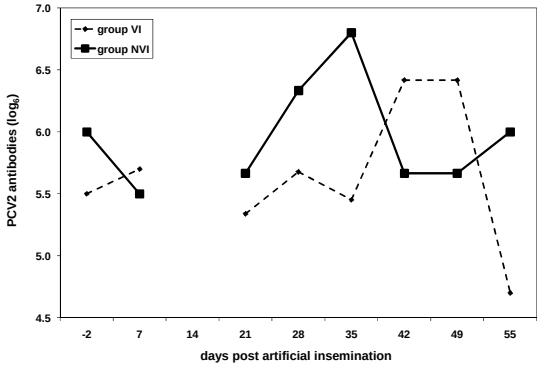

Fig. 1: Mean titres (log_6) of serum antibodies to PCV2 in gilts per group at different days post AI.

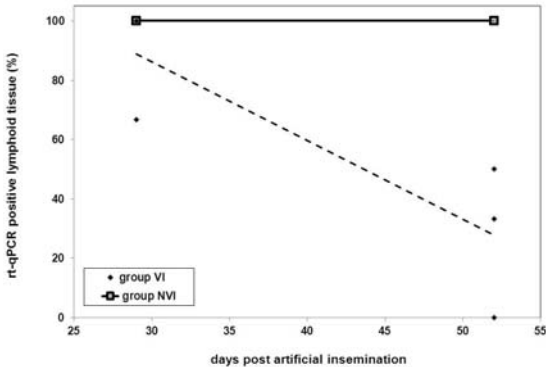

Fig. 2: Proportion of PCV2 real-time PCR-positive lymphoid tissue at the two sampling times.

Serology: No statistically significant difference (P>0.05) in the mean antibody titre against PCV2 was detected between the two groups at -2, 7, 21 and 28 DPAI. At 35 DPAI the mean antibody titre against PCV2 of group NVI pigs was significantly higher (P<0.05) than that of VI pigs, followed by a slight increase in mean antibody titre on 42 DPAI in group VI (Fig. 1).

All the animals had serum antibodies against PRRSV on -2 DPAI, but the antibody titres significantly decreased on *intra-mortem* sampling (P<0.05). Anti-ADV (anti-gB) and anti-PPV antibody titre showed a decreasing trend during the trial; no anti-ADV-gE antibodies were detected. Results indicated that no infection with PRRSV, ADV and PPV occurred during the trial.

PCV2 in blood, swabs and tissues: PCV2 DNA was detected in four gilt blood samples (NVI-2, NVI-3, NVI-5, NVI-6) at the beginning of the experiment (-2 DPAI).

Table 1: PCV2 assessment by real-time PCR in faecal, vaginal and nasal swabs, blood, lymphoid tissue set and reproductive tissues set

Sample	Group	Positive (%)		Negative (%)		Total	P
Faecal swab	VI	12	(28.6)	30	(71.4)	42	< 0.001
	NVI	38	(88.4)	5	(11.6)	43	
total		50	(58.8)	35	(41.2)	85	
Vaginal swab	VI	6	(14.3)	36	(85.7)	42	< 0.001
	NVI	25	(58.1)	18	(41.9)	43	
total		31	(36.5)	54	(63.5)	85	
Nasal swab	VI	13	(31.0)	29	(69.0)	42	<0,001
	NVI	29	(67.4)	14	(32.6)	43	
total		42	(49.4)	43	(50.6)	85	
Blood	VI	2	(5.7)	33	(94.3)	35	< 0.001
	NVI	21	(56.8)	16	(43.2)	37	
total		23	(31.9)	49	(68.1)	72	
Lymphoid tissues	VI	15	(41.7)	21	(58.3)	36	< 0.001
	NVI	36	(100)	0	(0)	36	
total		51	(70.8)	21	(29.2)	72	
Reproductive tissues*	VI	62	(39.5)	95	(60.5)	157	< 0.001
	NVI	121	(58.2)	87	(41.8)	208	
total		183	(50.1)	182	(49.9)	365	
Foeto-placental units **	VI	11	(13.1)	73	(86.9)	84	< 0.01
	NVI	35	(28.7)	87	(71.3)	122	
total		46	(22.3)	160	(77.7)	206	

*Included ovaries, oviduct, uterus, cervix, vagina, foetuses, placenta and amnios; **Data obtained considering the total of foetuses+placenta+amnios samples (feto-placental units).

Table 2: Comparison between VI and NVI groups of genomic load (expressed as Log_{10} genomic copies/mL) for swabs, blood and tissue samples (reproductive tissue set and lymphoid tissue set).

Sample	Genomic load (mean Log_{10} PCV2 genomic copies/mL)		P
	Group VI	Group NVI	
Faecal swab	0.00	4.32	< 0.001
Vaginal swab	0.00	3.50	< 0.001
Nasal swab	0.00	3.86	< 0.001
Blood	0.00	2.98	< 0.001
Lymphoid tissues	3.96	10.12	< 0.001
Ovary	4.04	6.57	0.014
Oviduct	5.26	6.93	0.038
Uterus	4.59	5.18	0.012
Cervix	3.76	6.46	0.11
Vagina	4.50	6.80	0.030
Foeto-placental units*	0.71	1.31	0.020
Tonsil	4.53	10.45	< 0.001
Uterine lymph node	3.70	9.22	< 0.001
Inguinal lymph node	3.12	9.89	< 0.001
Total (all samples)	2.51	4.24	< 0.001

*Data obtained considering the total of foetuses+placenta+amnios samples (feto-placental units).

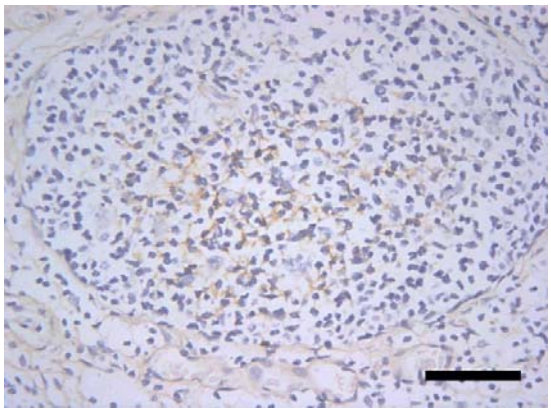

Fig 3: NVI-1, left tonsil. PCV2 immunohistochemistry. The lymphoid follicle shows a reticular labelling pattern (cytoplasmic localization in follicular dendritic cell of PCV2 antigen). DAB chromogen, haematoxylin counterstain. Obj 40x, Scale bar=50 µM.

Animals in the NVI group displayed a significantly higher proportion (P<0.001) of PCV2 viremic events (56.8%) throughout the trial, whereas the VI group animals were rarely viremic (5.7%; Table 1).

The proportion of total (nasal+ vaginal + faecal) PCV2-positive swabs was significantly lower (P<0.001) in the VI group (24.6%) than in the NVI group (71.3%; Table 1). Samples collected from VI gilts had a significantly lower genomic load than those collected from NVI animals, and was non-significant only in the comparison of the cervix data (Table 2).

In both groups (NVI and VI), data were available for two sampling times: 29 DPAI, i.e. the mean sampling time of the aborted/non-pregnant animals (3 NVI and 3 VI) and 53 DPAI, i.e. the mean sampling time of the pregnant gilts (3 NVI and 3 VI). Comparing the proportion of qPCR PCV2-positive lymphoid tissues in each group at the two sampling times (29 DPAI *vs* 53 DPAI), a decreasing trend was identified in the VI group (Fig. 2). The same trend was apparent even at analysing the sum of PCV2 genomic load of tissues belonging to two tissue sets compared between the two groups (Table 2).

Considering only pregnant animals, the comparison between the two groups showed a significantly higher PCV2 genomic load in foeto-placental units (foetal + amniotic + placental samples) of the NVI group (P<0.01). Only one foetal PCV2 positivity was detected in gilt NVI-3. PCV2 was found in the placenta of 11 out of 28 (39%, VI) and 31 out of 40 (77%, NVI) foetuses (P<0.01; Table 1).

The BLAST identified regions of local similarity between the PCV2b strain used for experimental infection and detected strain sequences higher than 98% only in one case out of 10 (99% homology) in a uterine segment adjacent to a foetus of gilt VI-5. The other nine sequences proved to be PCV2a strains. Moreover, the strain used for infection shared 99% similarity with Genebank sequence PCV2b HM038022.1.

Histopathology and immunohistochemistry: Histopathological hallmarks of PCVDs were absent. Only in six lymphoid tissues (tonsil and lymph node) of gilts NVI-1 and NVI-2 cytoplasmic immunolabeling to PCV2 was

detectable in scattered histiocytes and follicular dendritic cells (Fig. 3). Other recorded lesions were "background" lesions of conventional pigs: mild enzootic pneumonia or fibrosing pleuritis (n=5), mild interstitial hepatitis (n=2), mild erosions/ulcers on gastric pars oesophagea (n=2). No differences between groups were present. Other organs were grossly unremarkable and histology did not reveal any lesions.

DISCUSSION

This study was conducted in conventional gilts simulating the effect of PCV2 vaccination under field conditions. Already before infection (-2 DPAI), some of the animals used showed PCV2 viremia and all were PCV2 antibody-positive. The experimental infection with PCV2b-spiked semen was effective in inducing an increase in anti-PCV2 antibodies in both groups, similar to data reported with non-conventional sows (Madson *et al.*, 2009b). Recent field experiments, experimental trials and investigations supported the hypothesis that swine herds are infected by a swarm of PCV2 genotypes, which can cause prolonged viremia, reinfection, and coinfection, predisposing to recombination (Khaiseb *et al*., 2011; Gerber *et al.*, 2012). As PCV2 infections by different strains or genotypes may overlap in field conditions, there were also coinfections of different PCV2 strains in our experimental trial because PCV2a, prevalent and due to a spontaneous infection, were detected in addition to the strain used for experimental infection. Sequencing results confirmed the possibility of PCV2a/b coinfection (Zhai *et al.*, 2011) at least in some of the tested animals. Detection of the PCV2b genome used for the infection in the tested samples demonstrated the ability to induce a coinfection in subjects already positive for PCV2a.

Vaccination of gilts and sows aims also to reduce the spread of PCV2 in the breeding sector (Madson and Opriessnig, 2011). Vaccination efficacy should be viewed pragmatically as the capacity to hold and restrain the negative outcomes, shedding and viremia, that maximize the risk of horizontal and vertical transmission, mainly in the breeding sector. Most studies on the effect of PCV2 vaccination on sow performance sought to demonstrate a positive effect on reproductive parameters (reduced preweaning pig mortality, improved farrowing rates) (Joisel *et al.*, 2008; Schøning *et al.*, 2008; Maurin-Bernaud *et al.*, 2012) in field conditions, and only two addressed the experimental demonstration of *in utero* PCV2 replication (Madson *et al*., 2009b; Madson *et al*., 2009c). The PCV2 present in blood and swab sets (fecal, vaginal and nasal) from the present trial was significantly lower in vaccinated animals, confirming the role of the vaccine in reducing shedding contributing to decreased horizontal transmission.

The fact that four out of the 12 gilts were viremic before infection (-2 DPAI) is consistent with the widespread infection of conventional pigs with PCV2. Shedding mirrors viremia (Patterson *et al.*, 2011; Rose *et al.*, 2012) that was lower in vaccinated animals, a condition that reduced the probability of shedding and minimized vertical transmission. No vaccinated gilt was viremic on -2 DPAI, suggesting vaccination has a protective effect. The only PCV2 qPCR-positive foetus belonged to sow NVI-3, while in the VI group no foetal positivity was recorded. Sow NVI-3 displayed an intermediate antibody titre at -2 DPAI and was viremic throughout the trial. This finding supports the hypothesis that prolonged viremia is a source of foetal infection (Madson and Opriessnig, 2011) and the risk can be reduced by the vaccine's effect on viremia. Foetal infection arises by transplacental cross of PCV2. From this point of view placental infection is a prodromic event leading to foetal infection and the lower percentage of placentas positive to PCV2 in VI gilts should be considered a protective effect of the vaccine to prevent the foetal infection. A similar vaccine effect was shown in other studies in which conclude that PCV2 infection of naïve pregnant sows may not result in reproductive failure but can be associated with foetal infection alone (Madson *et al.*, 2009b; Madson *et al.*, 2009c).

Lymphoid compartments are privileged sites for PCV2 amplification. In this tissue set a decrease of genomic viral load was seen during the trial by comparing the percentages of positive samples collected from non-pregnant animals at 29 DPAI with those collected from pregnant animals at 53 DPAI. This viral clearance was identified in vaccinated individuals only, while the NVI group had higher concentrations at both 29 and 53 DPAI sampling times. Furthermore, PCV2 genomic viral load, regardless of the two moments, was higher in NVI. This could be interpreted as an effective PCV2 clearance due to vaccination in VI animals. One of the consequences of this viral clearance is the lower frequency of viremic events and PCV2 shedding in vaccinated gilts.

Conclusions: PCV2 vaccine seems to play an active role in reducing virus shedding and tissue viral load in sows. The experimental data reported here support the hypothesis that vaccination of gilts before artificial insemination with PCV2-spiked semen had a protective effect against both horizontal and vertical transmission. PCV2 vaccination in the pig breeding sector benefits *in utero* foetuses because of a reduction of PCV2 viremia in their dam.

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