Comparison of cytokine profiles in peripheral blood mononuclear cells between piglets born from *Porcine circovirus 2* vaccinated and non-vaccinated sows

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Abstract

This study was aimed to evaluate the effect of *Porcine circovirus 2* (PCV2) sow vaccination on cell-mediated immune responses in sows and their progeny. At 7 weeks before farrowing, fifteen PCV2 PCR negative pregnant sows with medium-low antibody values were selected and randomly distributed in two groups according to the antibody levels. Seven sows were vaccinated with a commercial PCV2 vaccine and eight were injected with phosphate-buffered saline at 6 and 3 weeks before farrowing. Blood samples were taken from sows and their piglets (n=90) during the study duration. PCV2 DNA and antibodies were tested in sera, and cytokine (IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 and IL-10) levels were assessed in supernatant from cultured peripheral blood mononuclear cells. All sows and piglets were negative by PCV2 PCR throughout the study. Significantly higher PCV2 antibody levels were detected in vaccinated sows after vaccination and in their offspring after colostrum ingestion compared to the non-vaccinated counterparts. Vaccinated sows did not show significant differences in cytokine secretion levels at farrowing compared to unvaccinated dams. In contrast, piglets from vaccinated sows had significantly higher levels of cytokines potentially linked to Th1 memory cells (IFN-γ and TNF-α) in comparison to the ones from non-vaccinated dams. In conclusion, PCV2 sow vaccination, apart from triggering a humoral immunity response in sows and their progeny, might be associated to an increased transfer of cell-mediated immunity from the dam to the piglet.

Keywords

Porcine circovirus 2 (PCV2); sow vaccination; maternally derived immunity

1. Introduction

Porcine circovirus 2 (PCV2) is the etiological agent of several clinical or subclinical forms known as porcine circovirus diseases (PCVD) (Allan et al., 2002). Among these, PCV2-subclinical infection (PCV2-SI) is nowadays the most prevalent, representing the highest proportion of the negative economic impact at farm level in comparison to the other PCVDs (Alarcón et al., 2013).

The benefits of dam vaccination on their progeny have been demonstrated in terms of reduction of PCV2-systemic disease (PCV2-SD) prevalence, viremia and PCV2 load in tissues (Opriessnig et al., 2010; Pejsak et al., 2010; Fraile et al., 2012; O'Neill et al., 2012). However, this strategy does not provide full protection against PCV2 infection in the offspring, as PCV2 vertical transmission in vaccinated sows can occur (Madson et al., 2009a, b; Hemann et al., 2014).

PCV2 vaccination elicits both humoral and cellular immune responses against PCV2 (Fort et al., 2009; Martelli et al., 2011; Seo et al., 2014). In sows, the goal of vaccination before farrowing, is the protection of the offspring by means of maternal immunity transfer through colostrum. Several studies have shown the maternal antibody transfer from sows to piglets (Kurmann et al., 2011; Fraile et al., 2012; Sibila et al., 2013; Oh et al., 2014; Dvorak et al., 2017). Nevertheless, the passive transfer of the PCV2-specific cellular immune response to the offspring has hardly been investigated. To our knowledge, only one peer-reviewed study has demonstrated that maternally derived colostral lymphocytes from PCV2 immunized sows may be transferred to the progeny (Oh et al., 2012). In that study, the participation of these lymphocytes in the adaptive immune response was measured by *in vivo* delayed type hypersensitivity (DTH)

responses, *in vitro* lymphocyte proliferation and the presence of PCV2-specific IFN-γ-secreting cells (IFN-γ-SCs) in new-born piglets. However, in this context, information on cytokine profiles in piglets after colostrum intake and the influence of sow vaccination on these profiles is not available. Therefore, the objective of the present work was to assess the effect of sow vaccination against PCV2 on humoral and cell-mediated immunity in sows and their offspring.

2. Material and methods

2.1. Farm selection

The study was conducted in a commercial farm with 1,060 sows (Large White x Landrace) located in Spain. This farm was a two-site herd with all-in/all-out management and 4-week batch farrowing system. PCV2 vaccination in sows and piglets had never been applied in the herd under study. Sows were routinely vaccinated against *Porcine reproductive and respiratory syndrome virus*, *Aujeszky's disease virus*, *Swine influenza virus*, *Porcine parvovirus*, *Erysipelothrix rhusiopathiae, Escherichia coli* and *Clostridium perfringens.* Piglets were vaccinated against *Mycoplasma hyopneumoniae* at 2 days pre-weaning. Weaning was performed at 3 weeks of age. Besides, PCVD clinical problems were never detected in this herd and the average of the reproductive parameters was within the Spanish one (www.bdporc.irta.es). Furthermore, no clinical signs of any other significant diseases were observed.

In order to evaluate the PCV2 antibody levels in the sow herd, blood samples from 30 sows from 2nd to 7th parity were taken and processed by ELISA (Ingezim Circo IgG 11.PCV.K1®, Ingenasa, Madrid, Spain). PCV2 antibodies were detected in 29 out of 30 (96.7%) sows, observing the highest ELISA S/P values in 5th parity sows (Figure 1).

93

94 2.2. Study design

95 Fifteen healthy sows (parity 3-4th) with the same expected farrowing day were selected
96 from the screened farm at 7 weeks pre-farrowing. These animals were individually ear-
97 tagged and bled. Blood samples were tested by conventional PCV2 PCR (Quintana et al.,
98 2002) and ELISA (Ingezim Circo IgG 11.PCV.K1®). All sows were PCR negative and
99 showed low-medium (ranging from 0.27 to 0.85) ELISA S/P values. At 6 weeks pre-
100 farrowing, sows were randomly distributed in two treatment groups according to S/P
101 values. Seven sows were vaccinated by intramuscular injection with 2 mL of a
102 commercial inactivated PCV2 vaccine (CIRCOVAC®, Ceva) at 6 and 3 weeks pre-
103 farrowing. In parallel, eight non-vaccinated sows received 2 mL of phosphate buffer
104 saline (PBS) at the same time points and by the same route. Animals with different
105 treatments were comingled in the same gestation pens as well as in the same farrowing
106 unit rooms. In sows, blood samples were taken in vacuum tubes by jugular venepuncture
107 at 6 weeks pre-farrowing and at the farrowing week (Table 1).

108

109 At birth, all piglets from litters of studied sows were ear-tagged and registered. Cross-
110 fostering was not allowed for the sows included in the study. At 48-72 hours after birth,
111 blood samples from six healthy and medium-sized piglets per litter were taken in tubes
112 without anticoagulant (n=90). In addition, from two of these six piglets selected per litter,
113 blood samples were also taken in heparinized vacuum tubes (n=30). Once in the
114 laboratory, blood samples in heparin tubes were immediately processed to obtain
115 peripheral blood mononuclear cells (PBMCs), while the ones in tubes without
116 anticoagulant were centrifuged at 750 g during 20 min to extract the sera. Sera were
117 aliquoted and stored at -20ºC until testing.

Any abnormality related to general state, condition of the skin, hair and mucosa, respiratory, digestive and nervous signs, and locomotive problems was registered at different time points (Table 1) in both sows and piglets. Housing conditions, feeding system, feed characteristics and health management remained consistent along the course of the trial, and were the same for both experimental groups. The present study was approved by the Ethics Committee for Animal Experimentation from the *Universitat Autònoma de Barcelona* and the Animal Experimentation Commission from the local government (*Dpt. de Medi Ambient i Habitatge* from the *Generalitat de Catalunya*; Reference 9402).

2.3. DNA extraction and conventional PCR

DNA was extracted from 200 µL of serum by using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer´s instructions. DNA obtained was suspended in 90 µL of elution solution. Then, PCV2 genome was detected by standard PCR (Quintana et al., 2002). Each extraction and PCR plate included negative and positive controls, where samples were replaced for diethylpyrocarbonate (DEPC)-treated water or known PCV2 infected sample, respectively.

2.4. Indirect ELISA for measuring anti-PCV2 IgG antibodies

All serum samples were tested by Ingezim Circo IgG 11.PCV.K1® assay (Ingenasa, Madrid, Spain). Optical density (OD) was measured at 450 nm by PowerWave XS reader (BioTek). Cut-off was established at 0.3 OD (± Standard Deviation [SD]) following kit´s instructions. ELISA results were expressed as mean S/P ratio (OD of sample/OD of positive control for each ELISA plate) ± SD.

143

144 2.5. Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

145 PBMCs were isolated from blood collected in heparinized tubes by density gradient
146 centrifugation using Histopaque® 1.077 (Sigma, Madrid, Spain). PBMCs were washed
147 and suspended in complete RPMI-1640 (Lonza, Barcelona, Spain) (cRPMI) plus 10%
148 foetal bovine serum (FBS) (Sigma, Madrid, Spain); cell viability was assessed with
149 Trypan blue staining. Then, PBMCs were seeded into 96-well plates (1 x 10^6 cells/well)
150 and incubated with baculovirus-expressed PCV2 Cap protein (final concentration per
151 well: 0.6 μg/mL); phytohemagglutinin (Sigma, Madrid, Spain) (final concentration per
152 well: 10 μg/mL) as a positive control; or cRPMI plus 10% FBS as a negative control for
153 24 h at 37°C in a 5% humidified CO_2 atmosphere. After incubation, plates were
154 centrifuged and cell culture supernatants were collected and stored at -80ºC until further
155 examination.

156

157 2.6. Multiplex immunoassay for the quantification of cytokines

158 PBMC supernatant samples were analysed using ProcartaPlex Porcine Cytokine &
159 Chemokine Panel 1 (Affymetrix, eBioscience, Vienna, Austria) according to the
160 manufacturer's instructions. This multiplex immunoassay uses Luminex® xMAP
161 technology for the quantification of 9 cytokines: IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β,
162 IL-8, IL-4, IL-6 and IL-10. The plates were read by a MAGPIX® analyser (Luminex
163 Corporation) and the cytokine levels were determined according to standard curves using
164 xPONENT® 4.2 software (Luminex Corporation). Then, for the final calculation of
165 PCV2-specific cytokine secretion (pg/mL), cytokine levels in supernatants from PBMCs
166 with medium (background) were subtracted from cytokine levels in supernatants from
167 PBMCs stimulated with PCV2 Cap protein.

168

169 2.7. Statistical analyses

170 Statistical analyses were carried out using StatsDirect v3.1.1. Kruskal–Wallis test was
171 used for comparisons of ELISA S/P values and cytokine levels between groups and
172 between sampling points. Significance level was set at $p \leq 0.05$.

173

174 **3. Results**

175 3.1. Clinical signs and detection of PCV2 DNA in serum samples from sows and piglets

176 No evident clinical signs were observed in sows or piglets throughout the study. All sows
177 (15 out of 15) and piglets (90 out of 90) were PCR negative during all the study duration.

178

179 3.2. Anti-PCV2 IgG antibody levels in sow and piglet serum samples

180 Mean S/P levels (± SD) in sows and their offspring for both treatment groups are
181 represented in Figure 2. From 7 weeks before farrowing to farrowing week, vaccinated
182 sows showed an increase of ELISA S/P values, resulting in significantly higher ($p<0.05$)
183 antibody levels compared to the ones from the non-vaccinated sows at farrowing.
184 Moreover, piglets from vaccinated sows also had significantly higher S/P values than the
185 ones from non-vaccinated counterparts at 48-72 hours of life.

186

187 3.3. Cytokine levels

188 3.3.1. PBMC supernatant samples in sows

189 PCV2-specific cytokine concentrations for sows of the two treatment groups are shown
190 in Figure 3. No statistically significant differences were observed when comparing
191 vaccinated and non-vaccinated groups at each sampling point (pre- and post- treatment
192 injection) in any of the tested cytokines.

193

194 3.3.2. PBMC supernatant samples in piglets

195 PCV2-specific cytokine values in piglets at 48-72 hours after birth are summarized in
196 Figure 4. Piglets born from vaccinated sows had significantly ($p \leq 0.05$) higher levels of
197 IFN-α, IFN-γ, TNF-α and IL-1β than the ones from control group. Regarding the IL-8,
198 very high values close to the upper detection limit of the technique were found in both
199 groups (without significant differences). In the rest of the cytokines (IL-12p40, IL-4, IL-
200 6 and IL-10), no significant differences were found between groups.

201

202 **4. Discussion**

203 The main goal of the present work was to describe the cytokine profiles in piglets born
204 from vaccinated sows in comparison to the ones from unvaccinated sows. In the present
205 study, piglets from vaccinated sows had significantly higher levels of IFN-γ, suggesting
206 that these animals had memory T cells able to produce IFN-γ upon stimulation with a
207 PCV2 antigen. These findings were correlated with a previously study (Oh et al., 2012),
208 where significantly higher levels of IFN-γ-SCs were observed in piglets from vaccinated
209 sows with regard to the ones from unvaccinated sows after colostrum ingestion. The
210 assessment of IFN-γ-SCs is commonly used to measure the cell-mediated immunity
211 response, mainly for the following reasons: 1) the levels of PCV2 specific IFN-γ-SCs
212 increase after infection and vaccination (Fort et al., 2009; Koinig et al., 2015); in fact,
213 PCV2 vaccination induces a long-lasting immunity sustained by memory T cells and IFN-
214 γ secreting cells that might participate in the prevention of PCV2 infection (Ferrari et al.,
215 2014); 2) IFN-γ-SCs are inversely correlated with PCV2 viral loads in serum (Seo et al.,
216 2012a; Seo et al., 2012b); and 3) these T cells are specific for both PCV2 *Cap* and *Rep*
217 proteins (Fort et al., 2010). However, to the authors' knowledge, information about

general cytokine profiles in piglets after colostrum ingestion is missing in the peer-reviewed literature.

In the present study, piglets from vaccinated sows also had significantly higher levels of TNF-α, IFN-α and IL-1β. Especially relevant is the case of TNF-α, since the production of TNF-α simultaneously with that of IFN-γ by T cells after PCV2 vaccination has been potentially correlated with protection (Koinig et al., 2015). In that study, the induction of PCV2-specific antibodies after PCV2 piglet vaccination was only observed in five out of 12 animals. However, at this time point, all vaccinated pigs showed IFN-γ/TNF-α co-producing T cells and all vaccinated piglets were fully protected against viremia after subsequent challenge (Koinig et al., 2015). Regarding the other two cytokines (IFN-α and IL-1β), these are not linked to memory T lymphocytes, since they are produced by other cell types (mainly macrophages and dendritic cells) primarily involved in innate immunity response (Chase and Lunney, 2012). Therefore, these significant differences observed between groups could be due to differences in the proportion of cell populations in PBMCs from each group, although these cellular subpopulations have not been tested in the present trial. Another more direct way to evaluate the transfer of immune cells would have been to analyse the colostrum, testing PCV2-specific cell subpopulations or cytokine (mainly IFN-γ) secretion, as previously described (Oh et al., 2012). However, given the difficulty of isolating colostral cells, the present study offers a more practical (PBMC isolation and stimulation) and quick (Multiplex immunoassay for the quantification of different cytokines at the same time) method to assess indirectly but apparently reliably the potential transfer of cellular immunity to the offspring.

242 On the other hand, the same cytokine profiles were tested in sows before and after
243 treatment application. The cell-mediated immune response after PCV2 vaccination has
244 been minimally investigated in sows. In a previously published trial (Oh et al., 2012),
245 vaccinated sows showed an increase in the proportions of T lymphocytes ($CD4^+$, $CD8^+$
246 and $CD4^+CD8^+$) at 1 day post-partum, attributing these changes to PCV2 vaccination.
247 This higher proportion of immunological T cells in vaccinated sows might be related to a
248 higher excretion of cytokines linked to memory response; however, in the present study,
249 although vaccinated sows showed higher levels in almost all tested cytokines with regard
250 to the non-vaccinated ones, these differences were not statistically significant, most
251 probably due to the low number of sampled sows (7-8 sows per group).
252
253 In order to complement the cellular immunity results, active (sows) and passive (piglets)
254 humoral immunity was also evaluated in this study. In sows, PCV2 vaccination twice
255 before farrowing produced an increase of antibody values in comparison to the non-
256 vaccinated counterparts. This humoral response after immunization was observed in other
257 studies when sows were vaccinated before mating or farrowing (Gerber et al., 2011; Sibila
258 et al., 2013). In the present work, vaccinated sows had higher levels of antibodies in blood
259 at farrowing. This fact triggered a greater transfer of maternal antibodies to the piglets,
260 which were evident after colostrum ingestion, as was also detected in an earlier study
261 (Kurmann et al., 2011).
262
263 In conclusion, PCV2 vaccination at 6 and 3 weeks pre-farrowing elicited high antibody
264 values in sows at farrowing and in their offspring. Moreover, piglets from vaccinated
265 sows had significantly higher levels of cytokines potentially linked to Th1 memory
266 response (IFN-γ and TNF-α), suggesting that this vaccination strategy may confer PCV2

specific cell-mediated passive immunity to the progeny. Nevertheless, more research is needed in order to study in depth the transfer of maternal lymphocytes through colostrum as well as the role of the cytokines secreted by them in the new-born piglet.

5. Conflicts of interest

Salvador Oliver-Ferrando is an employee of Ceva Santé Animale.

6. Author's contributions

SOF participated in the design of the study, was involved in the field trial, performed the laboratory tests (together with ID) and drafted the manuscript. ID, MS and JS collaborated in the study design, contributed to the coordination of the trial, participated in the data analysis and results interpretation. All authors critically read and contributed to the manuscript, approving its final version.

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10. Figure Legends

Figure 1. Individual PCV2 ELISA S/P results in serum samples from sows with different parity number prior to the start of the study (farm screening).

Figure 2. PCV2 ELISA S/P results (mean±SD) in serum samples taken from the sows included in the study and their offspring at different time points. Different letters in superscript mean statistically significant differences among experimental groups at each sampling point (a>b; $p<0.05$).

Figure 3. PCV2-specific cytokine secretion (pg/mL) in PBMC supernatant samples from sows. In each graphic, the two boxes from the left correspond to the first sampling point (6 weeks pre-farrowing); the two boxes from the right correspond to the second sampling point (farrowing). The "x" symbol indicates the mean. No statistically significant differences were observed among vaccinated (V) and non-vaccinated (NV) sows at each time point.

Figure 4. PCV2-specific cytokine secretion (pg/mL) in PBMC supernatant samples from piglets at 48-72 hours after birth. The "x" symbol indicates the mean. *Statistically significant differences ($p\leq0.05$) among piglets born from vaccinated (V) and non-vaccinated (NV) sows.