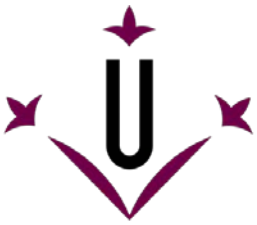

Effect of high and low levels of maternally derived antibodies on porcine circovirus type 2 (PCV2) infection dynamics and production parameters in PCV2 vaccinated pigs under field conditions

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Abstract:

The present study aimed to compare the efficacy of a porcine circovirus type 2 (PCV2) commercial vaccine in terms of average daily weight gain (ADWG) as well as infection dynamics in pigs with different maternally derived antibody (MDA) levels. A total of 337 animals from a PCV2 subclinically infected farm were distributed into two groups based on weight and PCV2 antibody levels (high [H] or low [L]) at 2 weeks of age. One week later, these animals were subdivided in four groups according to the treatment received. Vaccinated pigs (H-V and L-V) received 1 mL of a commercial vaccine and NV (H-NV and L-NV) received 1 mL of PBS. All piglets were subsequently bled at 7, 12, 18, 22 and 25 weeks of age and weighted at 12 and 25 weeks of age. V animals showed significantly lower PCV2 infection rates and viral load as well as higher ELISA S/P ratios and ADWG than NV ones. Compared with H-V piglets, L-V pigs showed numerically lower PCV2 infection rates, lower area under the curve of viral load, an earlier seroconversion and a numerically, but not significantly, higher ADWG. In this study, MDA did not seem to interfere with the effect of PCV2 vaccination on ADWG. However, only when a small subpopulation of pigs with the highest ELISA S/P ratios at vaccination was considered, an apparent interference of vaccine efficacy on ADWG was noticed. Therefore, the impact of the putative interference under field conditions is probably negligible for most farms.

Keywords: Interference; Maternally derived antibodies; Overcoming of maternal immunity; porcine circovirus type 2; Vaccine

1. Introduction

Porcine circovirus type 2 (PCV2) is the essential causative agent of a series of diseases known as porcine circovirus diseases (PCVD) [1, 2]. Postweaning multisystemic wasting syndrome (PMWS), currently denominated as PCV2-systemic disease (PCV2-SD), is one of the most economically important PCVD, since it increases the mortality rate and reduces production parameters [1, 3]. The effects derived from PCV2-SD have been drastically reduced by the use of different available commercial vaccines at the worldwide swine production market [4].

Besides the contrasted efficacy of PCV2 vaccines, some field and experimental studies have indicated that vaccination in face of high maternally derived antibody (MDA) levels may affect such efficacy. This potential interference has been studied at two different levels: vaccine-elicited humoral immune response and average daily weight gain (ADWG). In terms of humoral response, it has been proven that high antibody levels at the moment of vaccination jeopardize the seroconversion elicited by vaccination [5-8]. On the contrary, the effect of high MDA level on ADWG has only been assessed in three studies [5, 9, 10] in which the results obtained were not conclusive. In Fachinger et al. [10], animals included in the study were selected and separated in two groups based on the level of MDA at the moment of vaccination (>1:1000 and <1:1000 indirect fluorescence antibody [IFA] titres). Both groups of animals had similar ($p>0.05$) ADWG and in consequence it was concluded that this parameter was not affected by MDA level. However, the average titre for both groups of animals was not provided in the paper, and apparently they were not sharply different. Similarly, Fraile et al. [5] did not find statistically significant differences in terms of ADWG between 4-week-old vaccinated piglets derived from vaccinated and non-vaccinated sows. However, the correlation between initial MDA and ADWG (in the double

vaccinated ones) showed a negative slope, suggesting a potential negative effect when higher MDA titres were present at vaccination time. In Haake et al. [9], pigs were vaccinated at 1 or 3 weeks of age, which rendered different maturity of the immune system as well as levels of MDA at the moment of vaccination. In that study, animals vaccinated at 3 weeks of age had a higher ADWG than the ones vaccinated at 1 week of age. When compared, antibody titres of the pigs at 1 week of age were higher than those at 3 weeks of age.

Based on these inconsistencies, the present study aimed to assess PCV2 vaccination in terms of ADWG in purposely selected age-matched animals with high and low PCV2 ELISA S/P levels at the time of PCV2 vaccination. In addition, antibody and infection dynamics as well as viral loads of these animals were studied.

2. Materials and methods

2.1. Farm selection

The present study was conducted in a conventional Spanish multi-site production system in which PCV2 vaccination of 3 week-old piglets (Porcilis PCV, MSD) was applied routinely since 2 years before starting this study. An all-in-all-out management strategy was used in both nursery and fattening units.

In order to assess PCV2 infection before the start of the study, blood samples from 10 animals of different ages (5, 9, 14, 18 and 24 weeks of age) were taken. These blood samples were processed by standard PCR [11]. PCV2 genome was detected in 30% (3 out of 10) and 40% (4 out of 10) of pigs at 14 and 18 weeks of age, respectively. All tested samples from 5, 9 and 24 weeks of age were negative by PCR.

2.2. Study design

To ensure the presence of different levels (from very low to very high) of PCV2 MDA titres at the moment of vaccination, a proportion of sows were vaccinated before farrowing. Thus, from 64 randomly selected sows, 33 (52%) were vaccinated intramuscularly (IM) (neck muscle, needle length: 1.2x40 mm) with 1 dose of 2 mL of Circovac (Merial; batch number L383022) at 3 and 6 weeks pre-farrowing (V sows). The remaining 31 sows were left non-vaccinated (NV sows).

At 2 weeks of age, all healthy piglets (n=572) born from these 64 sows were ear-tagged, weighted and bled. Levels of PCV2 antibodies were measured by means of an indirect ELISA (detailed in PCV2 antibody detection section). The ELISA S/P ratios obtained in these 572 animals ranged from 0.14 to 2.68 (mean ± standard deviation [SD] =1.25±0.70). From all tested animals and based on the equivalences provided by Pileri et al. [12], those piglets with the highest (>1.44 or >log2 13 IPMA values, n=169) and the lowest (<0.96 or <log2 10 IPMA values, n=168) PCV2 ELISA S/P ratios were selected. Animals with medium (>0.96 and <1.44) PCV2 ELISA S/P ratios were removed from the study. Afterwards, selected animals were distributed based on their weight in 4 treatments groups according to the levels of MDA (H = High, L = Low) and vaccination status (V = vaccinated; NV = Non-vaccinated), as detailed in Table 1. At 3 weeks of age, V piglets (n=171) were injected IM (neck muscle; needle length: 0.9x25 mm) with 1 mL of Ingelvac Circoflex (Boehringer Ingelheim; Batch number 309-762B), in the right side of neck. NV animals (n=166) received the same dose of PBS at the same anatomic location. Animals from different treatments were comingled in the same pens, both in nurseries and fattening units. Mortality was recorded through the study.

During the study period, blood samples from all monitored pigs were subsequently taken at 7, 12, 18, 22 and 25 weeks of age. Once in the laboratory, blood samples were allowed to clot and centrifuged at 1500 g for 10 min.

Additionally, animals were weighted at 12 and 25 weeks of age. ADWG was calculated for the following periods: 2-12, 12-25 and 2-25 weeks of age. ADWG was calculated as the weight at the last studied time point minus the weight at first selected time point divided by the days lapsed between both time points.

Treatments, housing, and husbandry procedures were conducted in accordance with the guidelines of Good Experimental Practices, under the approval of the Ethical and Animal Welfare Committee of the Universitat Autònoma of Barcelona and Government of Catalunya (Protocol #DMAH-5796).

2.3. PCR and quantitative PCR (QPCR)

DNA extracted from serum samples was processed by standard PCV2 PCR and those yielding positive results were subsequently tested by a QPCR commercial kit (LSI VetMAX Porcine Circovirus Type 2 - Quantification). Standard PCR results were expressed as percentage of positive animals. QPCR results and area under the curve (AUC) of viremia [13] were expressed as $\log_{10}$ PCV2 DNA copies/mL (±SD) for QPCR positive samples.

2.4. PCV2 antibody detection

Serum samples were tested by a commercial indirect ELISA (INGEZIM, Circo IgG 1.1. PCV. K.1). Mean cut-off for this ELISA tests was set at 0.4 OD following the manufacturer's instructions. Results of ELISA were expressed as mean S/P ratio (±SD) and percentage of seropositive pigs.

149

150 *2.5. Statistical analyses*

151 All statistical analyses were done by SPSS 15.0 software (SPSS Inc., Chicago, IL,
152 USA). All the parameters assessed were compared at two different levels: 1) between V and
153 NV piglets, and 2) among H-V, L-V, H-NV and L-NV groups. Descriptive statistics were
154 used to summarize categorical and quantitative variables. Normality of distribution of the
155 examined quantitative variables was evaluated by Shapiro Wilk´s and Levene tests. Body
156 weight and ADWG were compared using an un-paired T-test. The Chi-square or Fischer
157 exact test was applied to evaluate the proportion of positive and negative animals by ELISA,
158 PCR and the mortality among these four groups. Data on ELISA S/P ratios, PCV2 viral
159 load and AUCs were assessed with a non-parametric Mann–Whitney test. The significance
160 level was set at 0.05.

161

162 **3. Results**

163 *3.1. Clinical findings*

164 No PCV2-SD-like clinical signs were observed throughout the trial (3 to 25 weeks
165 of age). Percentage of dead pigs was 3.2% (3 out of 93), 2.5% (2 out of 78), 2.6% (2 out of
166 76) and 4.4% (4 out of 90) for H-V, L-V, H-NV, L-NV animals, respectively ($P>0.05$).
167 Specific causes for such mortality were not investigated. In addition, 10 animals were
168 excluded from the study because of losing their ear tags.

169

170 *3.2. Comparisons between vaccinated and non-vaccinated pigs*

171 *3.2.1. PCR and QPCR*

172 PCV2 was firstly detected in both treatments at 18 weeks of age (Fig.1). Percentage

of PCV2 PCR positive pigs as well as mean PCV2 load in serum was significantly lower at 18, 22 and 25 weeks of age in V than NV pigs. PCV2 load AUC was significantly higher ($P < 0.05$) in NV (6.0±1.3 $\log_{10}$ PCV2 DNA copies/mL) than in V (4.8±1.1 $\log_{10}$ PCV2 DNA copies/mL) animals.

3.2.2. Antibody dynamics

At 7, 12 and 18 weeks of age, percentage of seropositive pigs was significantly higher in V group than in their NV counterparts (Fig. 2). Mean ELISA S/P values were significantly higher ($P<0.05$) in V compared to NV pigs from 7 to 18 weeks of age. From that moment onwards, the ELISA S/P ratios from V pigs were significantly lower ($P<0.05$) than those of NV animals.

3.2.3. Body weight and ADWG

No statistical differences were found in the body weight between V and NV piglets from the beginning to the end of the study (Table 2). ADWG was significantly higher ($P<0.05$) in V compared to NV during the 12-25 and 2-25 week-periods; specifically, V animals gained 33 and 17g per day more than NV pigs, in the respective periods.

3.3. Comparisons among vaccinated and non-vaccinated pigs with low and high ELISA S/P values

3.3.1. PCR and QPCR

A significantly ($P<0.05$) lower number of PCV2 PCR positive pigs was observed in L-V compared to NV groups at 18, 22 and 25 weeks of age and in H-V group compared to NV groups at 22 and 25 weeks of age (Fig 3). Between the two V groups, statistical

197 significant differences were only found at 22 weeks of age (higher number of PCV2 PCR
198 positive pigs in the H-V group).

199 A significantly ($P<0.05$) lower PCV2 load in serum was observed in L-V compared
200 to the both NV groups at 18 and 22 weeks of age and in H-V pigs compared to the NV
201 groups at 22 and 25 weeks of age. No statistical differences were found between L-V and
202 H-V groups throughout the study.

203 The AUC of viral load in H-V (5.1±1.3 $\log_{10}$ PCV2 DNA copies/mL) and L-V
204 (4.5±1.0 $\log_{10}$ PCV2 DNA copies/mL) groups was significantly lower ($P<0.05$) than in H-
205 NV (5.8±1.3 $\log_{10}$ PCV2 DNA copies/mL) and L-NV (6.2±1.3 $\log_{10}$ PCV2 DNA
206 copies/mL). However, no statistical differences were found between H-V and L-V ($P=0.09$)
207 and between H-NV and L-NV ($P=0.11$) AUC of viral loads.

208

209 *3.3.2. Antibody dynamics*

210 Statistically significant differences in percentage of ELISA positive animals among
211 the 4 groups were observed at 7, 12 and 18 weeks of age (Fig 4A). At 7 weeks of age, the
212 lowest ($P<0.05$) percentage of seropositive pigs was observed in L-NV, followed by the one
213 in L-V group. Five weeks later, L-NV group showed still a significantly lower ($P<0.05$)
214 percentage of ELISA positive pigs than the other three groups. At that point, while L-V and
215 H-NV had similar percentage of seropositive pigs, H-V group showed the highest rate of
216 ELISA positive pigs. At 18 weeks of age, the dynamics changed since the highest ($P<0.05$)
217 percentage of ELISA positive animals was observed in L-V animals.

218 A sharp decrease (up to 12 weeks of age) of ELISA S/P values was observed in both
219 H groups (Fig 4B). On the contrary, in the L groups the decrease in S/P values was seen
220 until 7 weeks of age. At that point, whereas L-V pigs showed a progressive increase of

ELISA S/P values, a flat line from 7 to 18 weeks of age was observed in L-NV ones. Afterwards, all groups experienced an increase of ELISA S/P ratios being significantly higher ($P<0.05$) in both NV groups than their V counterparts. At the two latter sampling points, L-V pigs had significantly lower ($P<0.05$) ELISA S/P ratios than H-V ones.

3.3.3. Body weight and ADWG

At 2 and 12 weeks of age, no significant differences were observed in body weight among the 4 groups (Table 2). At 25 weeks of age, L-NV showed the lowest body weight, being significantly lower ($P<0.05$) when compared to V pigs.

L-V and L-NV pigs showed the highest and the lowest ADWG values, respectively, in both periods 12-25 and 2-25 weeks. Statistically significant differences were observed between L-V and NV groups for the period 12-25 weeks and between V and L-NV for the period 2-25 weeks.

4. Discussion

The effect of MDA levels at vaccination age was assessed on ADWG as primary outcome. The initial hypothesis was that the higher the MDA at vaccination timing, the lower the ADWG. However, such hypothesis was not confirmed since a potential detrimental effect of MDA on ADWG was not evident. Although L-V animals grew 2 and 18 g per day more than H-V ones in the 2-25 and 12-25 week periods, such differences were not statistically significant. Besides, virological and serological parameters were also studied. In the present study, pigs vaccinated with low MDA seemed to take more benefit of the treatment than their counterparts with high MDA, since they had a lower PCV2 infection rate (at 22 weeks of age), lower AUC of viral load and showed an earlier

245 seroconversion (evident at 12 weeks of age). These latter results would be in accordance
246 with those previously published studies [5, 6, 8] in which the interference of high MDA
247 titres at the moment of vaccination with the humoral response elicited by the vaccine was
248 demonstrated. It is worthy to highlight, however, that vaccination was able to overcome
249 such interference since statistically significant differences were seen between H-V *vs* H-NV
250 animals in terms of infection rate at 22 and 25 weeks of age and mean ELISA S/P ratios at
251 18 weeks of age.

252 The specific reason by which MDA affected PCV2 virological and serological
253 parameters but not ADWG remains unknown. Recent data in non-vaccinated pigs have
254 demonstrated that the higher the AUC of viral load, the lower the ADWG [13]. This
255 situation applied in the present work when comparing the V and NV groups, but the
256 scenario is more complex when studying existing subpopulations in terms of low and high
257 MDA at vaccination. Under the scenario of low MDA levels, V animals had significantly
258 lower AUC and significantly higher ADWG than their NV counterparts; on the contrary, in
259 a high MDA level context, V animals had a significantly lower AUC but similar ADWG
260 than NV ones. In addition, the numeric but non-significant ADWG differences between L-
261 V and H-V may suggest that, if occurring, interference of MDA with ADWG would be seen
262 only in those animals with extremely high MDA levels. This hypothesis would be
263 supported by the fact that in the present and in Haake et al. [10] studies, the best (although
264 no significantly different) productive performances were seen when vaccination was
265 applied in the presence of low MDA titres. Indeed, in the present study, the 10 animals with
266 the highest MDA titres (>2.4 ELISA S/P titres) at the moment of vaccination, coming all of
267 them from vaccinated sows, grew 52 g/day less than the rest of the vaccinated animals (n=
268 151, with average ELISA S/P value of 1.23±0.65) (data not shown). According to Pileri et

al. [12], these >2.4 S/P values would be equivalent to >17 log_2 immunoperoxidase monolayer assay (IPMA) titres. In fact, the MDA titres producing interference on the humoral response to vaccination has been established around 8-10 log_2 IPMA titres [7], being 14 log_2 IPMA the result of the highest dilution of the IPMA test routinely performed [14]. In consequence, 17 log_2 IPMA titres would be an extremely high MDA titre, probably not very frequently found under field conditions. Therefore, if these high MDA titres are present in a very small proportion of animals, the economic relevance of such putative interference would be presumably low or negligible in most of the cases.

These very high antibody titres were "artificially" created by means of vaccinating a proportion of the sows. This action was aligned with the need of a sufficient number of piglets with the highest MDA levels possible to achieve the objective of this study. It cannot be ruled out that both humoral and cellular immunity linked to the colostrum intake from these sows might have exerted certain effect on the obtained results. However, such effect is not very likely, since MDA levels reached the lowest S/P ratios around 12 weeks of age, while evidence of PCV2 infection started at 18 weeks of age. In consequence, it is difficult to believe that, at those ages, MDA exerted an effect on virus dynamics. Moreover, the antibody evolution of piglets with high antibody values coming from vaccinated and non-vaccinated sows were very similar (data not shown), reinforcing the notion that sow vaccination did not apparently bias the obtained results. The potential effect of sow vaccine-derived cellular immunity on piglet vaccine response was not known. According to the results obtained in field and experimental studies [8, 15], such effect is probably short-lasting and not likely to interfere on piglet vaccine intake. Moreover, it is also unlikely that such immunity would exert effects on pigs that were infected in the growing-finishing phase. However, cell-mediated immunity was not measured in the present study and no

conclusions can be drawn.

5. Conclusion

Under the conditions of this study, vaccination at 3 weeks of age was able to efficiently control PCV2 infection, reduce PCV2 viral load, increase the serological response against the infection and improve ADWG when compared to NV pigs. Although the pigs with the best growth performance were those with low ELISA S/P values at the moment of vaccination, presence of high MDA values at that moment did not interfere in the ADWG of pigs. Evident detrimental effects of MDA on ADWG were exclusively observed in a minimal number of pigs with extremely high MDA at the time of vaccination, which probably represents a negligible population of animals under field conditions.

Conflict of interest statement

None of the authors declares conflict of interests that could inappropriately influence or bias the content of the study.

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361

Table 1

Piglet distribution according to PCV2 MDA level at 2 weeks of age, PCV2 vaccination (V= vaccinated; NV= Non-vaccinated) and sow treatment (V= vaccinated; NV= Non-vaccinated).

PIGLETS		**Sow treatment**		
Level of S/P ratio at 2 weeks of age	**Treatment**	**NV**	**V**	**Total**
High S/P ratio (> 1.44, equivalent to >log_2 13 IPMA values*)	**NV**	6	70	76
	V	13	80	93
Low S/P ratio (< 0.96, equivalent to <log_2 10 IPMA values*)	**NV**	75	15	90
	V	59	19	78
Total		153	184	337

Table 2

Body weight (mean, [kg ± SD]) at different weeks of age and average daily weight gain (ADWG, [g ± SD]) for different week intervals. Different letters within a sampling point mean statistically significant differences ($p<0.05$).

		Body weight (kg)				ADWG (g)		
	Weeks of age	2	12	25	Period (weeks)	2-12	12-25	2-25
Groups	NV	2.4±0.5 [a]	27.1±5.8 [a]	98.3±13.9 [a]	Groups	324.5±72.1 [a]	774.5±111.7 [a]	570.9±81.5 [a]
	V	2.4±0.5 [a]	26.8±4.9 [a]	101.0±12.8 [a]		321.4±60.9 [a]	807.4±107.6 [b]	587.5±75.3 [b]
Groups	H-NV	2.5±0.5 [a]	28.0±5.8 [a]	100.9±12.8 [ab]	Groups	336.5±72.3 [a]	792.3±90.9 [a]	586.1±74.7 [ab]
	L-NV	2.4±0.5 [a]	26.3±5.7 [a]	96.2±14.6 [a]		314.4±70.8 [a]	759.7±125.2 [a]	558.2±85.0 [a]
	H-V	2.4±0.5 [a]	27.5±5.2 [a]	101.3±13.0 [b]		329.9±655.3 [a]	799.0±112.7 [ab]	586.8±81.7 [b]
	L-V	2.4±0.6 [a]	26.1±4.4 [a]	100.5±12.6 [b]		311.7±54.1 [a]	816.8±101.5 [b]	588.3±67.8 [b]

Figure captions

Fig.1. PCR and QPCR results for V and NV. Percentage of PCV2 PCR positive pigs (bars and left Y axis) and $\log_{10}$ PCV2 DNA viral loads (mean± SD) (lines and right Y axis) of PCR positive pigs in V and NV groups at the six sampling time points, respectively. In the table, different low-case letters within a sampling point mean statistically significant differences in the percentage of PCR positivity between V and NV pigs ($P<0.05$); different capital letters within a sampling point mean statistically significant differences in PCV2 DNA load in serum between V and NV pigs ($P<0.05$).

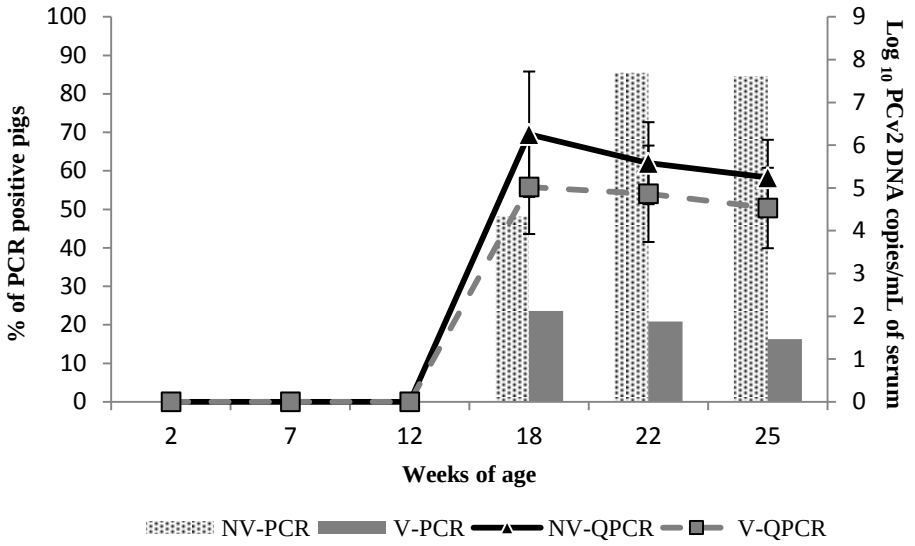
Fig. 2. ELISA results for V and NV. Percentage of ELISA positive pigs (bars and left Y axis) and PCV2 ELISA S/P ratio (mean± SD) (lines and right Y axis) values and in the six sampling points for both V and NV pigs, respectively. Different low-case letters in the table within a sampling point mean statistically significant ($P<0.05$) differences in percentage of ELISA positivity between V and NV animals; different capital letters within a sampling point mean statistically significant differences in ELISA S/P values among the 4 groups ($P<0.05$).

Fig. 3. PCR and QPCR results for H-NV, L-NV, H-V and L-V. Percentage of PCV2 PCR positive pigs (bars and left Y axis) and $\log_{10}$ PCV2 DNA loads (mean± SD) (lines and right Y axis) of PCR positive pigs in H-NV, L-NV, H-V and L-V groups at the six sampling times, respectively. In the table, different low-case letters within a sampling point mean statistically significant differences in the percentage of PCR positivity among the 4 groups ($P<0.05$); different capital letters within a sampling point mean statistically significant differences in PCV2 DNA load in serum among the 4 groups ($P<0.05$).

Fig. 4. A. ELISA positive percentage for H-NV, L-NV, H-V and L-V. Percentage of ELISA positive pigs at the six sampling points for H-NV, L-NV, H-V and L-V pigs. Different low-case letters in the table within a sampling point mean statistically significant ($P<0.05$) differences in percentage of ELISA positive pigs among the 4 groups. **B.** ELISA S/P ratio for H-NV, L-NV, H-V and L-V. PCV2 ELISA S/P ratio (mean± SD) values at the six sampling points for H-NV, L-NV, H-V and L-V pigs. Different low-case letters in the table within a sampling point mean statistically significant ($P< 0.05$) differences in ELISA S/P values among the 4 groups.

Figures

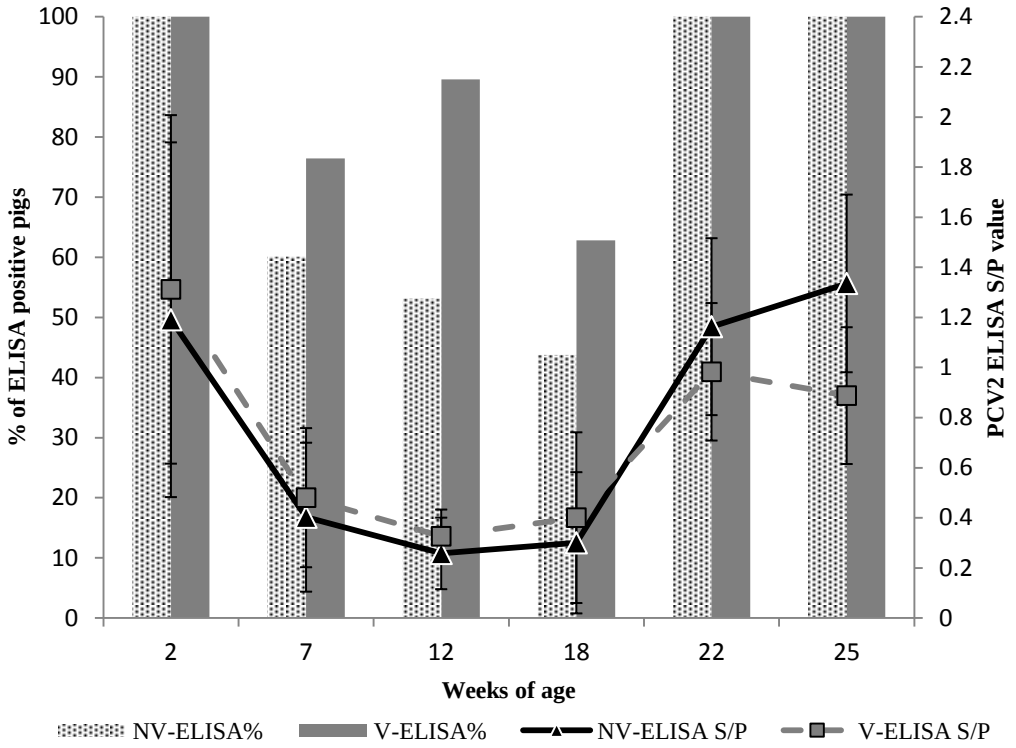
Fig. 1.



	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	load	%	load	%	load	%	load	%	load	%	load
NV	a	A	a	A	a	A	a	A	a	A	a	A
V	a	A	a	A	a	A	b	B	b	B	b	B

Figures

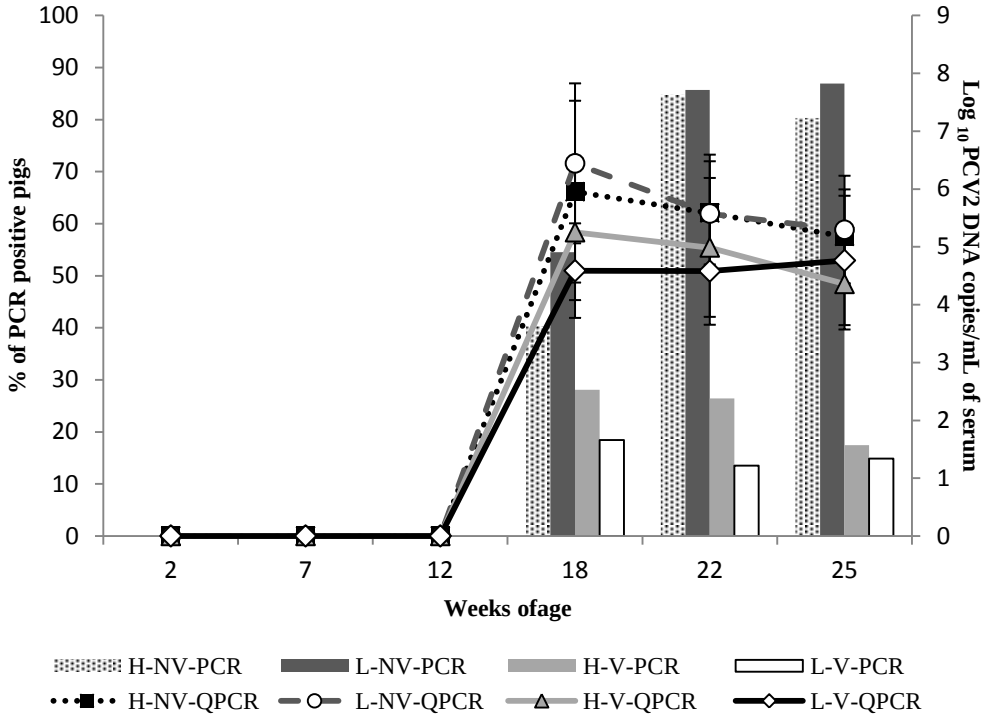
Fig. 2.



	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	S/P	%	S/P	%	S/P	%	S/P	%	S/P	%	S/P
NV	a	A	a	A	a	A	a	A	a	A	a	A
V	a	A	b	B	b	B	b	B	a	B	a	B

Figures

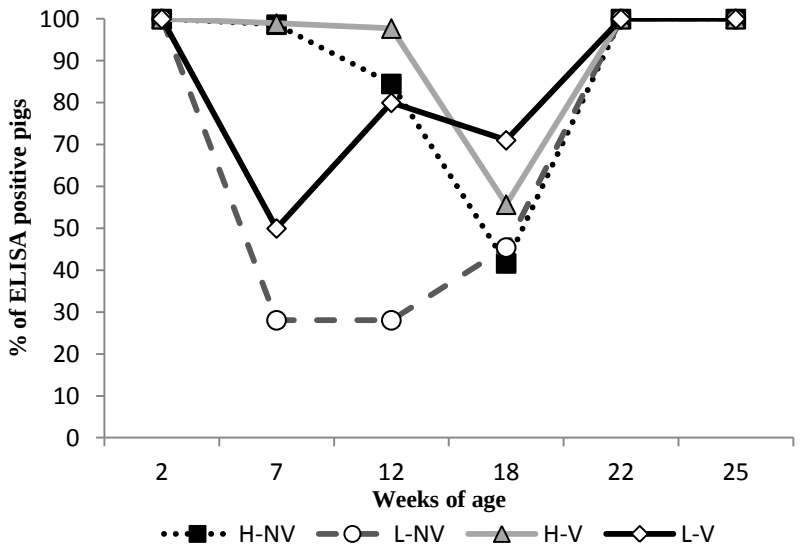
Fig. 3



	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	load	%	load	%	load	%	load	%	load	%	load
H-NV	a	A	a	A	a	A	ab	AB	a	A	a	A
L-NV	a	A	a	A	a	A	a	A	a	A	a	A
H-V	a	A	a	A	a	A	bc	BC	b	B	b	B
L-V	a	A	a	A	a	A	c	C	c	B	b	AB

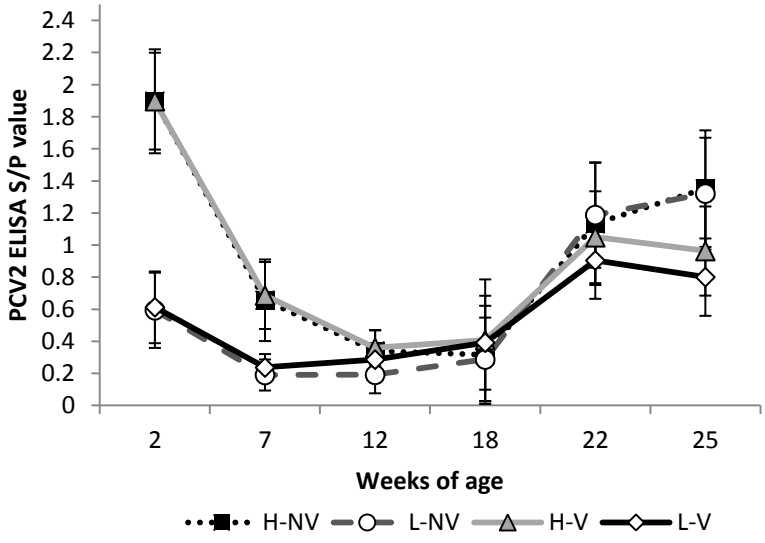
Figures

Fig. 4A.



	2 weeks	7 weeks	12 weeks	18 weeks	22 weeks	25 weeks
H-NV	a	a	a	a	a	a
L-NV	a	b	b	a	a	a
H-V	a	a	c	a	a	a
L-V	a	c	a	b	a	a

Fig. 4B.



	2 weeks	7 weeks	12 weeks	18 weeks	22 weeks	25 weeks
H-NV	a	a	a	a	ab	a
L-NV	b	b	c	a	a	a
H-V	a	a	a	b	b	b
L-V	b	b	b	b	c	c