Assessment of neutralizing and non-neutralizing antibody responses against Porcine circovirus 2 in vaccinated and non-vaccinated farmed pigs

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Vaccination is the most efficacious procedure to curtail Porcine circovirus 2 (PCV2)-associated diseases (PCVAD). Experimental studies indicate that PCV2 vaccine-induced virus-neutralizing antibodies play a major role in protection from PCVAD. However, the immune response to PCV2 vaccination of pigs on farms is less clear. Analysing groups of age-matched vaccinated and non-vaccinated farmed pigs, we found significantly increased levels of virus-neutralizing antibodies only in vaccinated pigs belonging to the age group with the highest risk for developing PCVAD. Serum levels of PCV2 genomes were not different between corresponding age groups. Levels of antibodies directed against a linear peptide from the PCV2 capsid protein correlated with those of virus-neutralizing antibodies and reached the highest levels in older, non-vaccinated animals, pointing towards an intense interaction between PCV2-infected cells and the immune system. In conclusion, current PCV2 vaccines are in need of improvement to induce stronger and more rapid immunity to prevent PCV2 infection.

Since the 1990s, Porcine circovirus 2 (PCV2) has been recognized as one of the most significant pathogens in pig production globally (Ellis, 2014; Segalés et al., 2013). PCV2 is the smallest autonomously replicating virus known. It consists of a circular ssDNA genome with three to four major open reading frames (ORFs) and one structural protein, the capsid protein (Finsterbusch & Mankertz, 2009). PCV2 is aetiologically involved in several syndromes affecting pigs, together known as PCV2-associated diseases (PCVADs). Of these, post-weaning multisystemic wasting syndrome (PMWS) of 5–12-week-old piglets is the most prevalent and devastating disease (Rose et al., 2012). PCVADs are characterized by PCV2-induced functional and structural defects of the immune system (Kekarainen et al., 2010). In non-vaccinated pig herds of modern production, the morbidity and mortality of PMWS can reach over 20% (Horlen et al., 2008). Vaccination with commercially available PCV2 vaccines leads to enhanced performance of vaccinated pigs in pig production. This is reflected by reduced mortality, stronger weight gain and shortened time periods to market (Beach & Meng, 2012; Kristensen et al., 2011). Experimental vaccination studies have demonstrated that the levels of PCV2 genome copies in serum after PCV2 challenge are significantly lower in vaccinated than in non-vaccinated animals (Cline et al., 2008; Fort et al., 2008). This correlates with elevated PCV2-neutralizing antibodies in serum (Fort et al., 2007), suggesting that these antibodies prevent higher levels of circulating PCV2 genomes. As high PCV2 load is an essential component for clinical disease (Harding et al., 2008; Olvera et al., 2004), it has been suggested that the levels of PCV2-neutralizing antibodies are an indicator of protection against PCVAD (Fort et al., 2007; Trible et al., 2012).

The immunological response to PCV2 under production conditions is less clear: whilst there is little doubt about the effects of vaccination on curtailing PCVAD, PCV2 viraemia is neither consistently prevented nor reduced by vaccinating farmed pigs (Gerber et al., 2012). This study served to enlighten the role of PCV2 neutralizing and non-neutralizing anti-PCV2 antibodies of pigs from modern pig production facilities. We examined 160 pigs from 13 pig farms in Canada. Eighty animals had been vaccinated using different types of commercial PCV2 vaccines following the instructions of the vaccine manufacturers. Serum samples were further classified into four groups according to the age of the donor pig at the time point of sampling (suckling pigs: 0–21 days of age; nursery/weaned pigs: 22–90 days of age; nursery/weaned pigs: 91–150 days of age; nursery/weaned pigs: >150 days of age). We measured the levels of virus-neutralizing antibodies against PCV2 and monitored the levels of anti-PCV2 peptides. The results showed significantly increased levels of virus-neutralizing antibodies only in vaccinated pigs belonging to the age group with the highest risk for developing PCVAD. Serum levels of PCV2 genomes were not different between corresponding age groups. Levels of antibodies directed against a linear peptide from the PCV2 capsid protein correlated with those of virus-neutralizing antibodies and reached the highest levels in older, non-vaccinated animals, pointing towards an intense interaction between PCV2-infected cells and the immune system. In conclusion, current PCV2 vaccines are in need of improvement to induce stronger and more rapid immunity to prevent PCV2 infection.
pigs: 21–84 days of age; finisher/grower pigs: 84–180 days of age; sows/boars: more than 180 days of age). Levels of PCV2 genome in serum were determined by assessing PCV2 genome copy numbers with real-time quantitative PCR (qPCR). DNA extraction from 100 μl of serum was performed using a Mag-Bind Viral DNA/RNA kit (Omega Bio-tek). The master mix was prepared using PCV2 primers (forward: 5′-CTGACTGTGGTTCCGTAGAT-3′; reverse: 5′-GTTTACCGCTGGAGAGGATCAAG-3′), a PCV2 Taq-Man probe (5′-ATGTAATTACTGCTCCGCGCATACATCG-3′), nuclease-free water (Ambion), 2 × buffer and 25 × enzyme (Ambion AgPath-ID One-Step RT-PCR). A standard curve was constructed by log10 dilutions of a plasmid construct of PCV2. The qPCR program used was 45 °C for 10 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 45 s (Bio-Rad CFX96 Real-time PCR). Results were log10 transformed and reported as PCV2 DNA copy number ml⁻¹. Based on the limit of detection of the qPCR assay, a sample was considered positive when the result was equal to or higher than 10³ PCV2 DNA copies (ml sample)⁻¹; only positive samples were considered for statistical analysis of the qPCR assay. A virus neutralization assay was performed to determine neutralizing antibody titres in serum. Inactivated swine serum (25 μl) was threefold serially diluted (1 : 3 to 1 : 6561), incubated with 3.1 × 10⁸ TCID₅₀ PCV2 and added to porcine kidney cells (PK-15) for 48 h. Cells were fixed and stained against the virus capsid. Neutralizing antibody titres were determined as described previously (Eschbaumer et al., 2014; Supplementary Methods S1, S2, and S3, available on the online Supplementary Material); results were log₂ transformed before analysis. An indirect ELISA was performed as described previously (Trible et al., 2011) to measure the levels of antibodies against a linear peptide of PCV2 capsid (169STIDYFQPNNKR180).

Statistical analyses of data were performed on duplicate test results for each sample and dilution using SPSS v.17.0 software. The Kolmogorov–Smirnov test was performed to test the normality of distribution. If the distribution was normal (virus neutralization assay results), Student’s t-tests were performed to analyse the differences between PCV2-vaccinated and non-vaccinated animals of the same age group. One-way ANOVA was performed to analyse the results between different aged but identical vaccination groups; if the result was significant, post-hoc analysis was performed using Tukey’s HSD test with Bonferroni adjustment. If the distribution of data was not normal (qPCR and ELISA results), Mann–Whitney U tests were performed to analyse the differences between results of PCV2 vaccinated and non-vaccinated animals of the same age group. A Kruskal–Wallis one-way ANOVA was performed to analyse the results between different aged but identical vaccination groups; if the result was significant, the analysis was followed by Dunn’s multiple comparison test. Spearman’s correlation test was performed using neutralizing antibody titres and sample : positive (S : P) ratio values of the ELISA. A χ² test for association was performed to compare the animal’s vaccination status and the prevalence of PCV2 DNA copies ml⁻¹ for each age group. A P value of ≤0.05 was considered significant.

Only vaccinated suckling animals had a significant association between vaccination and low prevalence of PCV2 DNA genome copies (χ²=10.63, P=0.001). We found no significant difference in the levels of viral load of PCV2 PCR-positive animals when comparing PCV2 vaccinated with non-vaccinated pigs (P>0.05). Thus, in this and another study (Gerber et al., 2012) but not in the study by Han et al. (2013), the protective effects of PCV2 vaccination were not reflected by reduced PCV2 serum load under field conditions.

Levels of PCV2 genome copies were significantly different between different aged but identical vaccination group (P<0.05). Post-hoc analysis demonstrated that nursery/weaned vaccinated animals had significantly higher genome copy numbers compared with grower-finishers (P<0.01) and sows/boars (P<0.06; Fig. 1). Non-vaccinated sows and boars had significantly lower levels of PCV2 genome copies compared with nursery-weaned (P<0.05) and grower-finishers (P<0.05; Fig. 1). From all the age groups, nursery/weaner pigs were at the highest risk for developing PCVAD (Rose et al., 2012). The high levels of PCV2 in sera from nursery/weaned pigs are probably due to declining maternal antibodies, weak adaptive immunity and permanent exposure to PCV2 through the environment, in addition to other typical age- and production-related stress factors (Rose et al., 2012).

As vaccinated pigs are significantly protected from developing PCVAD (Beach & Meng, 2012; Kristensen et al., 2011; Martelli et al., 2011), and as the levels of PCV2-neutralizing antibodies were found to correlate inversely with viraemia in experimental (Meerts et al., 2006) but not field (Fort et al., 2007) studies, we determined levels of PCV2-neutralizing antibodies in our cohort. A newly established virus neutralization assay allowed us to calculate PCV2 neutralizing antibody titres based on analyses of more than 5000 cells and a minimum of 500 infectious foci (cells) per serum dilution (Supplementary Method S4). Images of PCV2-infected cells were captured using an InCell 2000 analyser high-content screening system (GE Healthcare) and analysed with an InCell Analyser 1000 Workstation (GE Healthcare; Supplementary Method S2). This method provides greater accuracy over traditional immunofluorescence-based virus neutralization assays (Fort et al., 2007) because of the increased sampling, objective determination of positive foci and standardization of image exposure capture settings (Eschbaumer et al., 2014; Supplementary Method S5). Statistical analyses were performed on all samples as described above.

Vaccinees belonging to the high-risk group (nursery/weaner pigs) represented the only one of the four age groups that had significantly higher PCV2-neutralizing antibodies compared with their non-vaccinated
counterparts (P<0.05; Fig. 2). In all other groups, no significant differences in PCV2-neutralizing antibody levels between vaccinated and non-vaccinated pigs were observed. This result indicated that vaccination had a positive impact on PCV2-neutralizing antibody induction in the group of pigs with the highest risk for developing PCVAD.

As the titres of PCV2 neutralizing antibodies in vaccinated pigs were not significantly higher than those of non-vaccinated pigs in the other age groups, one could speculate that, under field conditions, PCV2 neutralizing antibodies need assistance from other host defence mechanisms to successfully prevent PCVAD and reduce the amount of infectious circulating virus and PCV2-infected cells. As protection from PCVAD is induced by PCV2 vaccination (Beach & Meng, 2012; Kristensen et al., 2011; Martelli et al., 2011), it is plausible that, under field conditions, PCV2-specific T-cell immunity is most likely an essential and substantial component of protection from PCVAD.

This conclusion is supported by results obtained in other studies (Fort et al., 2009b; Martelli et al., 2011).

PCV2 neutralizing antibody titres were significantly different between animals of different aged but identical vaccination groups (P<0.05). Post-hoc analysis demonstrated that older, vaccinated animals, i.e. those belonging to the grower/finisher and sow/boar groups, were significantly higher than those of younger animals (P<0.00 and P<0.01, respectively; Fig. 2). As most of the older animals had substantial titres of PCV2 neutralizing antibodies, it appears likely that sows transfer some of these antibodies to piglets via colostrum and milk. This would explain the low PCV2 viral load of the suckling piglets belonging to vaccinated group 4 and three samples of non-vaccinated group 1 are missing due to insufficient amount of serum to perform the PCR test.

<table>
<thead>
<tr>
<th>PCV2*</th>
<th>V</th>
<th>NV</th>
<th>V</th>
<th>NV</th>
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<td>%</td>
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<th>Grower/ finisher</th>
<th>Sow/ boar</th>
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<td>Non-vaccinated</td>
<td>Median ± IQR</td>
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<tr>
<td>Vaccinated</td>
<td>Non-vaccinated</td>
<td>Mean ± SD</td>
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Fig. 1. Levels of PCV2 genome copies in sera from qPCR-positive pigs grouped into four age categories and further divided into vaccinated (V) and non-vaccinated (NV) animals. The results of individual samples are depicted as open symbols; the median ± interquartile range (IQR) are denoted in the graph. Based on our statistical ANOVA, groups of animals that showed a significant difference (P<0.05) are indicated by an asterisk in the graph. The prevalence of qPCR-positive animals is demonstrated in the table below the graph. Based on our statistical analysis for association, a significant association in the age group is shown as a superscript letter A in the table. The results from two samples of vaccinated group 4 and three samples of non-vaccinated group 1 are missing due to insufficient amount of serum to perform the PCR test.

Fig. 2. Titres of PCV2-neutralizing antibodies in sera from pigs grouped into four age categories and further divided into vaccinated (V) and non-vaccinated (NV) animals. The results of individual samples are depicted as open symbols; means ± SD are denoted on the graph. Based on our statistical ANOVA, groups of animals that showed a significant difference (P<0.05) are indicated by an asterisk on the graph. The prevalence of virus neutralization assay-positive animals is shown in the table below the graph.
influenza virus (Gorres et al., 2011). As pigs are capable of generating substantial titres of PCV2 neutralizing antibodies within 2–5 weeks under experimental conditions (Seo et al., 2012), the lengthened time period needed to produce substantial titres of PCV2-neutralizing antibodies by ‘field’ pigs must thus be due to an environmental and not an intrinsic factor like host or viral genetics. We propose that subclinical infection with PCV2 – which may affect most piglets perinatally (Gerber et al., 2012) – hampers a timely and strong development of PCV2 neutralizing antibodies.

It has been suggested that non-neutralizing antibodies directed against the linear PCV2 epitope represented by aa 169–180 of the capsid protein ‘dive’ the humoral immune response away from generating antiviral, i.e. neutralizing antibodies (Trible et al., 2011). Employing the same method as described previously (Trible et al., 2011), we found antibodies directed against this epitope in almost all animals (Fig. 3a). We found a significant difference in the levels of antibodies in animals of different aged but identical vaccination group (P<0.05). Vaccinated animals belonging to the nursery/weaner group had significantly higher aa 169–180 antibody levels than pigs belonging to the sows/boars group (P<0.01; Fig. 3a). In non-vaccinated pigs, antibody levels (against aa 169–180) of growers/finishers and of sows/boars were significantly higher than those of suckling (P<0.00 and P<0.01, respectively) and nursing/weaned pigs (P<0.00 and P<0.01, respectively; Fig. 3a). The antibody titres against aa 169–180 of non-vaccinated growers/finishers and sows/boars were significantly higher than of their vaccinated counterparts (P<0.05 and P<0.00, respectively; Fig. 3a). The stronger antibody response against aa 169–180 in older non-vaccinated pigs was probably due to repeated or persisting exposure of this linear epitope to antigen-presenting cells, B- and T-lymphocytes. As vaccination alone did not lead to an increased level of non-neutralizing aa 169–180-specific antibodies in all the age groups – as indicated by the results obtained in the vaccinated animals – this immunogenic peptide must have originated mainly during PCV2 replication and not from the vaccine. The release of this PCV2 peptide from infected cells could have happened in the process of PCV2-induced cell killing or as a consequence of an attack of CD8$^+$ T-lymphocytes on PCV2-infected cells. In either case, a higher antibody level against this peptide probably reflects a more intense interaction between PCV2 and the immune system of non-vaccinated animals than occurs in vaccinated pigs.

We found a positive correlation between the titres of neutralizing and (non-neutralizing) antibodies against aa 169–180 (r=0.42; P<0.00; Fig. 3b); however, we were unable to verify any inverse correlation between these types of antibodies as suggested in other studies (Trible et al., 2011). It thus appears that the hypothesis that the aa 169–180 linear peptide is a decoy epitope does not hold true for farmed pigs. We instead suggest

\begin{align*}
\text{ELISA}^* & \quad V \quad NV \\
% & 14/20 \quad 0/9 \\
& 70 \quad 0 \\
\text{ELISA}^* & \quad V \quad NV \\
& 16/20 \quad 20/20 \\
& 80 \quad 100 \\
\text{ELISA}^* & \quad V \quad NV \\
& 20/20 \quad 20/20 \\
& 100 \quad 100 \\
\text{ELISA}^* & \quad V \quad NV \\
& 20/20 \quad 31/31 \\
\end{align*}

\[ y=0.06x + 0.09 \]

\[ r = 0.42 \]

\[ y = 0.06x + 0.09; r^2 = 0.13 \]
that antibodies against aa 169–180 may serve as an indirect marker for the extent of PCV2 replication in tissues, although further studies would need to be carried out to test this possibility.

Our study is not the first to demonstrate the absence of efficacy of PCV2 vaccination in terms of reducing PCV2 serum load in farmed pigs (Gerber et al., 2012). Our results are in contrast to those from experimental PCV2 vaccine studies (Seo et al., 2012). This discrepancy could be due to lower infectious 'pressure', i.e. less infectivity and/or limited exposure to PCV2 in experimental setups than exists in pig production. It is also likely that farmed pigs experience a higher level of immunosuppression at the time around weaning than experimental pigs do. We propose that subclinical infection with PCV2 before or shortly after weaning plays a major role for compromising functions of the immune system, although clinical outcomes seem to occur later. In conclusion, only subclinical infection with PCV2 deserves more attention and research, together with efforts to improve current PCV2 vaccines.

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