One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions

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1. Introduction

Since the 1990s, porcine circovirus type 2 (PCV2) has been considered the causative agent of the post-weaning multisystemic wasting syndrome (PMWS), one of the major swine diseases worldwide (Allan et al., 1999). Currently, PCV2 is also associated with other diseases, such as porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex, enteritis, and reproductive failure, collectively referred to as porcine circovirus diseases (PCVD) (Segälés et al., 2005). Epidemiologic investigations have shown that porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), Haemophilus parasuis,
Actinobacillus pleuropneumoniae, Streptococcus suis, and Mycoplasma hyopneumoniae are most commonly involved in this syndrome (Chae, 2004). The effects of the virus on the pig immune system have not been fully elucidated. The development of PMWS has been related to an activation of the immune system (Krakowka et al., 2001) or it has been suggested that PCV2 infection may cause immunosuppression, when PMWS occurs (Krakowka et al., 2002; Darwin et al., 2004). Several studies reported that PCV2 infection induces apoptosis of lymphoid cells leading to extensive depletion of lymphocytes.

The control of PCVD is based on management strategies, control of coinfections, and vaccination. The first available commercial PCV2 vaccine is based on an inactivated virus and is registered for use in gilts and sows (Reynaud et al., 2004). At present, three commercial PCV2 vaccines are available for use in piglets from 3 to 4 weeks of age. Two of these vaccines are based on the PCV2 Cap protein expressed in a baculovirus system (Fachinger et al., 2008; Fort et al., 2008) and the third is based on a chimeric inactivated virus containing the genomic backbone of the nonpathogenic PCV type 1 but replacing its ORF2 capsid gene by that of PCV2 (Fenaux et al., 2004). The induction of an active immune response against the ORF2-encoded capsid protein has been demonstrated to be the major immunogenic mechanism of protection (Nawagitgul et al., 2002; Blanchard et al., 2003).

Under field conditions, all PCV2 vaccines currently available on the market are effective, showing decreased mortality and cull rates and significantly improving average daily weight gain (ADWG), concomitantly with a decrease of the frequency of coinfections in herds affected with PMWS (Cline et al., 2008; Desrosiers et al., 2009; Fachinger et al., 2008; Horlen et al., 2008; King et al., 2008; Kixmoller et al., 2008; Opríešnig et al., 2008a,b; Tacker et al., 2008; Segalés et al., 2009). PCV2 vaccines are thought to reduce viremia burden and viral-induced specific lymphoid lesions but little is yet known about the mechanism by which they are able to elicit protective immunity (Fachinger et al., 2008; Fort et al., 2008; Horlen et al., 2008). Some studies indicate that the effects are related to total and neutralising antibody responses as well as to cell-mediated immunity (Larochelle et al., 2003; Fort et al., 2008, 2009a; Kixmoller et al., 2008; Opríešnig et al., 2008a,b). Most of the published reports have characterised the humoral immunity to PCV2 infection based on the detection of total anti-PCV2 antibodies, showing seroconversion that occurs either in subclinically or non-PMWS-infected and PMWS-affected pigs (Rodríguez-Arrioja et al., 2000; Sibila et al., 2004; Grau-Roma et al., 2009). Conversely, the role and mechanisms of the adaptive cell-mediated immune response on controlling PCV2 infection and related diseases have not been studied in depth, particularly under field conditions. Previous reports based on laboratory trials describe that cell-mediated immunity measured by the number of PCV2-specific interferon-γ (IFN-γ) secreting cells (SC) may play a role in mediating viral clearance in combination with neutralising antibodies (Fort et al., 2009a,b) and that the intensity of the cell-mediated immune response may be influenced by the load and the extent of viral replication. These aspects are worth investigating under field conditions both in diseased pigs naturally infected by PCV2 in combination with coinfections and in vaccinated animals showing no or few clinical signs.

The present study was designed to investigate the efficacy of a one-dose PCV2 subunit vaccine based on the PCV2 Cap protein expressed in a baculovirus system at two different farms with the presence of PCVD by measuring morbidity, mortality, ADWG (average daily weight gain), carcass weight, and PCV2 viremia load. Vaccine immunogenicity was assessed in terms of development of humoral (total antibodies) and cell-mediated (PCV2-specific IFN-γ secreting cells) immune responses. Serology to PRRSV and M. hyopneumoniae was also performed.

### 2. Materials and methods

#### 2.1. Selection of farms

The study was conducted in the Northern part of Italy at two farms selected based on their history of PMWS. In both herds, at approximately 15–20 weeks of age, clinical signs of PWMS characterised by wasting, respiratory signs, and growth retardation were mainly associated with a marked increase in the mortality rate. The diagnosis fulfilled the internationally accepted disease case definition, including clinical signs, gross lesions, histopathologic findings, and presence of PCV2 in lymphoid lesions (Segalés et al., 2005). Seropositivity to PCV2 in all categories of animals (replacement gilts, sows, nursery pigs, growers, and fatteners) was demonstrated. Before the start of the study, 5 wasted pigs were autopsied at each farm and the diagnosis was reconfirmed.

Farm 1 was a 900-sow farrow-to-finish herd that, in the previous year, experienced a 6% and an 8% mortality rate in the nursery and fattening periods, respectively. This farm was seronegative for Aujeszky’s disease virus (ADV) and seropositive for PRRSV, M. hyopneumoniae and A. pleuropneumoniae. Low titres of antibodies at HI to SIV were obtained from some samples and were inconclusive.

Farm 2 was a three-site farm with 1850 sows experiencing a 2% and a 10% mortality rate in the nursery and fattening periods, respectively. Seropositivity to PRRSV and M. hyopneumoniae and seronegativity to ADV were found.

Sows of both herds were vaccinated for Aujeszky’s disease (3 times/year), porcine parvovirus and erysipelas (both at mid-lactation). Piglets were vaccinated for Aujeszky’s disease according to the National Control Program.

#### 2.2. Experimental design

This study was a double-blind, randomised, controlled field trial performed according to the principles of “Good Clinical Practice” and included 818 piglets (males and females). The day before inclusion, piglets were identified, double ear-tagged and assigned to the two treatment groups [unvaccinated = placebo/control (group A) and PCV2-vaccinated (group B)] as they came to hand sequentially (A–B–A–B–A–B…). The sequential allocation was continued over the litters. The identification of the sow and the date of birth of the piglets were recorded.
At inclusion (weaning day, 21 ± 3 days of age), vaccinated animals (group B) received one dose of a commercial PCV2a-based subunit vaccine (Porcilis PCV®; Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) containing the PCV2 capsid (Cap) protein expressed in a baculovirus system suspended in an α-tocopherol + liquid paraffin-based adjuvant administered intramuscularly (2 mL) in the right neck muscle according to the recommendation of the manufacturer. The same amount of adjuvant was injected in the same anatomic location in control nonvaccinated pigs (group A). The administration of vaccine and placebo was performed using a double-blind fashion system for both farms. Animals of both groups were injected at weaning and moved to the nursery units. Table 1 lists the details of the study animals at the time of inclusion.

After weaning, pigs from both treatment groups were kept in mixed groups until the end of the trial, when animals were sent to the slaughterhouse (at approximately 9 months of age). Treatments, housing, husbandry, and feeding were conformed to the European Union Guidelines and identical for both experimental groups at each farm. Administration of concomitant treatments (injections) was used as a parameter to evaluate morbidity.

Dead animals or those that had to be euthanised for reasons of animal welfare were recorded daily.

In both herds, at inclusion (day of vaccination, 3 weeks of age), blood samples were collected from 2 piglets of each pluriparous sow litter: one for each treatment group, up to 44 (22 piglets for each treatment group). These piglets were identified by a progressively numbered ear tag.

Moreover, in piglets from farm 1, blood samples were taken at 4 (+1 week post-vaccination (PV)), 5 (+2 weeks PV), 6 (+3 weeks PV), 7 (+4 weeks PV), 9 (+6 weeks PV), 12 (+9 weeks PV), 15 (+12 weeks PV), 16 (+13 weeks PV), 17 (+14 weeks PV), 18 (+15 weeks PV), 19 (+16 weeks PV), 20 (+17 weeks PV), 22 (+19 weeks PV), 26 (+23 weeks PV), and 35 (+32 weeks PV) weeks of age.

In farm 2, blood samples were collected at vaccination (3 weeks of age), at 4 (+1 week PV), 6 (+3 weeks PV), 12 (+9 weeks PV), 16 (+13 weeks PV), 18 (+15 weeks PV), 20 (+17 weeks PV), 22 (+19 weeks PV), 24 (+21 weeks PV), 26 (+23 weeks PV), and 35 (+32 weeks PV) weeks of age.

2.3. Gross pathology, histopathology, and immunohistochemistry studies

The objective of the pathologic studies was to establish the PMWS diagnosis in all pigs from both farms that died, those needing euthanasia, and runts during the entire study period. These pigs underwent gross pathologic examination and histopathology within 24 h. Samples from inguinal, mesenteric and mediastinic lymph nodes were removed from necropsied pigs and fixed in 10% buffered formalin. Fixed samples were processed for routine histopathology and 5-μm thick sections were stained with hematoxylin and eosin to be examined for lesions compatible with PMWS. The diagnosis of PMWS was made when all three criteria of the accepted international individual case definition for the disease (clinical signs, mainly wasting, moderate-to-severe lymphoid lesions, moderate-to-high amounts of PCV2 in those lesions) were present. The amount of PCV2 in tissue samples was also assessed by real-time quantitative PCR (qPCR) using the methods reported by Olvera et al. (2004).

Immunohistochemistry for detection of PCV2-specific antigen was performed on formalin-fixed and paraffin-embedded sections of inguinal, mediastinal, and mesenteric lymph nodes using a rabbit polyclonal antiserum (Sorden, 2000). PCV2 antigen scoring was done by a pathologist in a blind manner using the score range in accordance with Opriessnig et al. (2004).

2.4. DNA extraction and qPCR to detect PCV2 nucleic acid in tissue samples and serum

To detect and quantify the PCV2 DNA by PCR, DNA was firstly extracted from 200 μL of serum or 200 μL of 1:10 phosphate-buffered saline (PBS) homogenate of lymph node tissue, by using TRizol LS (Invitrogen, San Diego, CA, USA) following the manufacturer’s instructions. The DNA obtained was suspended in 50 μL of diethylpyrocarbonate-treated water. Real-time qPCR was carried out using a LightCycler 1.5 (Roche, Basel-CH).
2.5. Serology

2.5.1. Detection of PCV2 antibody titres

The anti-PCV2 antibody titres in sera were determined using a blocking enzyme-linked immunosorbent assay (ELISA). The wells of microtitre plates were coated overnight at 2–8 °C with baculovirus-expressed PCV2 ORF2 antigen. Subsequently, the plates were washed and blocked with casein buffer at 37 °C for 1 h. After washing, serial 4-fold dilutions of the test sera were added. An internal standard serum and a positive and negative standard serum were run in parallel in each plate. The sera were incubated for 1 h at 37 °C and the plates were then washed before the addition of a PCV2-specific biotinylated monoclonal antibody (mAb). After 1 h incubation at 37 °C, plates were washed again and incubated for 45 min at 37 °C with avidin-labeled horseradish peroxidase (APO; DAKO A/S, Glostrup, Denmark). After washing, a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 min at room temperature. The reaction was stopped by the addition of 4 N sulphuric acid and the extinction was read in a photometer fitted with a 450-nm filter (Titertek Multiscan Plus MK 11–Titertek Instruments Inc., Huntsville, AL, USA) within 15 min after the reaction was stopped. The raw data were processed and titres were calculated using the Multi-calc program with a cut-off extinction value set at 50% blocking. The cut-off extinction was calculated from the positive and negative standard serum and titres were expressed as log2.

2.5.2. Detection of antibody titres to other infections

The presence of antibodies to PRRSV and sample/positive (S/P) ratio were determined using a commercially available ELISA kit (HerdChek® Porcine Reproductive and Respiratory Syndrome Antibody Test Kit, IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer’s instructions. The Herd Check® test bases the sample classification on the S/P ratio, which is defined as (sample OD – negative control OD)/(positive control OD – negative control OD). Sample to positive control ratios >0.4 were considered positive. Antibodies to M. hyopneumoniae were evaluated by a commercially available ELISA test (Herd Check® M. hyopneumoniae, IDEXX Laboratories). The presence of antibodies to gE glycoprotein of ADV was measured using a commercially available ELISA kit [HerdChek® PRV g1 (gE) test kit, IDEXX Laboratories] according to the manufacturer’s instructions. A commercial ELISA kit (CHECKIT-APP-ApxIV ELISA test kit, IDEXX Laboratories) was used for the detection of antibodies against A. pleuropneumoniae. Serology to Swine Influenza was performed by using HI.

2.5.3. Determination of PCV2-specific IFN-γ secreting cells (SC)

The frequency of IFN-γ SC in the peripheral blood of pigs was determined according to Martelli et al. (2009). Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1.077® gradient and plated at 2 × 10⁵ cells/well in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) into 96-well plates (MultiScreen® HTS-IP MSIP54510 – Millipore) coated overnight at 4 °C with 10 μg/mL anti-pig IFN-γ mAb (P2G10, BD, Biosciences, Franklinlakes, NJ, USA) and blocked with RPMI-1640 + 10% FBS for 2 h at 37 °C. For the in vitro antigen recall, a whole PCV2 strain (112/11) at 0.1 multiplicity of infection (MOI) was used as stimulus, in RPMI-1640 + 10% FBS, for 20 h at 37 °C, 5% CO₂. In all samples, PBMC were >98% viable as confirmed by Trypan blue exclusion. Afterward, plates were incubated for 1 h at 37 °C with 0.5 μg/mL anti-pig IFN-γ biotin-labeled mAb (P2C11, BD) and then with 1:750 alkaline phosphatase-conjugated anti-biotin mAb in PBS + 0.5% BSA. Plates were finally incubated for 7 min with a BCIP/NBT solution (BioRad, Hercules, CA, USA) and the reaction was stopped with distilled water. The frequency of PCV2-specific IFN-γ SC was determined using an AID® ELISpot Reader (AID® ELISpot Software v.3.5). As a positive control, 1 × 10⁵ PBMC/well were incubated with phytohemagglutinin (10 μg/ml); as a negative control, 2 × 10⁴ PBMC were incubated in the absence of antigen (mock stimulus: supernatant of non-PCV2-infected PK-15 cells). The background values (number of spots in negative control wells) were subtracted from the respective counts of the stimulated cells and the immune responses were expressed as number of IFN-γ SC per million PBMC (IFN-γ SC/10⁶ PBMC).

2.6. Statistical analysis

To estimate the effect of vaccination on the probability of a pig of becoming viremic, a mixed effect logistic regression model was fitted to take into account the non-independence of the repeated measures on the same subjects and the effect of the sow (litter effect). These two variables were treated as “random effects” in the model, whereas the effect of the farm (two levels), sex, time, and treatment and their interactions were considered as “fixed effects”. The effect of the time × treatment interaction was highly significant (P = 0.0146), indicating that these results are not attributable to chance alone. The package “lme4” was used (Bates and Sarkar, 2007; lme4: linear mixed-effects models using S4 classes, R package version 0.999375-32).

To estimate the effect of vaccination on the probability of a pig suffering from PCVD and being lost, considering the competing risks of dying or being lost from other causes, a stratified Cox proportional hazard model was fitted, according to Putter et al. (2007) and Therneau and Grambsch (2000). The model accounted also for the “cluster” effect of the sow (“litter effect”) because piglets from the same litter are expected to have similar clinical histories.

The effect of the vaccination on weight gain was evaluated within a mixed effects model, given the hierarchical structure of the experiment (Pinheiro and Bates, 2000). Vaccine efficacy was measured by the proportion of cases that it prevented, comparing disease outcome in the treated versus control groups. Efficacy was
presented here as one-risk ratio (the so-called preventive fraction), which gives the risk in the vaccinated group as a proportion of the risk in the control group (Kirkwood and Sterne, 2003).

Humoral and cellular immunity, determined as titres of anti-PCV2 antibodies and frequencies of IFN-γ SC respectively, were statistically evaluated by using ANOVA (analysis of variance) and Dunnett’s test in order to highlight differences between treatment groups and changes over time within the same group throughout the experiment.

3. Results

3.1. Reduction of morbidity and mortality in PCV2-vaccinated animals

In both herds, clinical signs potentially compatible with PMWS were mainly observed during the fourth and fifth month after vaccination (weeks 16–23 PV). Morbidity was quantified by recording the number of individual antimicrobial treatments (injections) during the duration of this experiment. As shown in Fig. 1, unvaccinated animals received more injections during the entire duration of the study; according to the statistical analysis using a Poisson model, the differences were significant (P < 0.0001). Specifically, animals belonging to group A (placebo/controls) are expected to receive 30% more injections as compared to vaccinated pigs [95% confidence interval (CI95%): 16–50%].

Dead pigs, animals needing euthanasia, and runt non-marketable pigs (lost pigs) underwent pathologic and virologic investigations to be categorised as PMWS or non-PMWS using the recognised diagnostic criteria. The mortality rates in both groups were comparable before the onset of viremia and all dead animals were not affected by PMWS.

At 15–16 weeks of age and onward in both herds, mortality was associated with PCR positivity to PCV2 and with macroscopic and microscopic lesions referred to as PMWS using the pathologic criteria. The details of the occurrence of mortality categorised as PMWS or non-PMWS on a weekly basis at both farms are shown in Fig. 2.

Among the animals that had to be euthanised or removed because they were runts, after the onset of PCV2 viremia they were removed from the study because of wasting (15 vs. 0 pigs; placebo/control vs. vaccinated), growth retardation (12 vs. 3 pigs, respectively), locomotory disorders (4 vs. 5 pigs, respectively), and intestinal torsions (1 vs. 2, respectively).

Before the onset of viremia, total losses were 7.3% and 7.8% and after PCV2 viremia 9.02% and 0.2%, respectively, in the placebo/control and vaccinated groups. Overall, considering both herds for the study duration, total losses (dead, euthanised, and runts) were 16.03% and 8.0% in the placebo/control and vaccinated groups, respectively. The estimated hazard ratio for losses related to PMWS in group B (vaccinated animals) compared with group A (placebo/control) was 0.082 (CI95%: 0.030–0.229; P < 0.0001; Fig. 3). Under the conditions of this study, according to a stratified Cox proportional hazard model accounting for the non-independence of repeated measurement of the same subject and the effect of the sow (litter effect) and for the competing risks of dying from other causes, the probability of a pig vaccinated with a single dose of the test vaccine at 3 weeks of age suffering from PCVD/PMWS was 12 times less than an unvaccinated control pig. The overall efficacy of the vaccine administered, expressed as preventive fraction was 0.918 (CI95%: 0.771–0.970). The preventive fraction represents and provides the proportion of cases that can be prevented with vaccination compared to the unvaccinated population.

3.2. ADWG and carcass weight at slaughterhouse

The ADWG is considered a parameter for measuring the effect of PMWS either in acute or in subclinical cases. Table 2 shows the ADWG in vaccinated and placebo/control animals for the intervals among the three different weighing time points. The ADWG in the first time period (3–12 weeks of age) was not significantly different. Differences in the ADWG between the treatment groups were observed during the subsequent time period (12–26 weeks), when the vaccinated animals had 70 g/day higher weight gain than placebo/control animals (P < 0.001). Moreover, the proportion of animals whose body weight was at least 25% lower than the mean body weight of the respective treatment group at 26 weeks of age was 6.5% and 2.6% in placebo/control and vaccinated groups, respectively.

Carcass weights in pigs from farm 2 were recorded at the slaughterhouse, as shown in Table 2. In vaccinated animals, the average carcass weight was 4.5 kg higher than in placebo/controls (P < 0.012).

3.3. PCV2 viremia

The course of PCV2 viremia at both farms is shown in Fig. 4. A sudden onset of viremia was observed at 16–17 weeks of age at both farms. In farm 1, peak levels of 95–
100% of PCR-positive blood samples from placebo/control animals were reached when animals were 20–22 weeks old. A decline of PCR positivity was detected at 26 weeks. The majority of samples with high viral loads (>10^6 DNA copies/mL serum) were observed at 19–22 weeks of age (Fig. 4a), and 70% of the animals had at least one blood sample with a viral load >10^7 DNA copies/mL. Conversely, in the vaccinated group, the proportion of viremic pigs was significantly lower compared to the placebo/control group; between weeks 19 and 22 of age, 40% of the animals were

![Diagram](image)

Table 2
ADWG in PCV2-vaccinated (group B) and placebo/control (group A) for two intervals between three different weighing time points during two study periods and carcass weight at slaughter.

<table>
<thead>
<tr>
<th></th>
<th>A (placebo)</th>
<th>B (vaccinated)</th>
<th>A – B (difference)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–12 weeks of age</td>
<td>481</td>
<td>478</td>
<td>+3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>12–26 weeks of age</td>
<td>811</td>
<td>881</td>
<td>−70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CARCASS (kg)</td>
<td>140.5</td>
<td>145.0</td>
<td>−4.5</td>
<td>&lt;0.012</td>
</tr>
</tbody>
</table>

* Carcass weight with head left on.
Fig. 3. Probability of a pig in the placebo/control (A) and PCV2-vaccinated (B) groups to be lost because of PCVD and other causes according to the estimated hazard risk over time, in both farms.

Fig. 4. Course of viremia over time in placebo/control (a) and PCV2-vaccinated pigs (b) of farm 1 and farm 2. Data are expressed as number of PCV2 DNA copies/ml of serum.
viremic, with a viral load never \( > 10^6 \) DNA copies/mL serum (Fig. 4b). At farm 2, the peak of viremia was observed at 18–20 weeks of age with 95% PCR-positive pigs; pigs with a high viral load ranged from 55% to 60% (Fig. 4c). A viral burden \( > 10^7 \) DNA copies/mL was detected in 42% of blood samples from the controls. In the vaccinated group only one blood sample was PCR positive at 18 weeks of age, with a low viral burden \( (< 10^6 \) DNA copies/mL; Fig. 4d).

The present data clearly indicate that vaccination against PCV2 induced a statistically significant reduction in the proportion of viremic animals and also of the viral load in the blood for both farms in this study \((P < 0.001)\).

### 3.4. Serology

#### 3.4.1. Serologic response to PCV2 vaccination and infection

The course of serology for PCV2 in both farms is shown in Fig. 5. At inclusion (vaccination day), pigs of both groups had comparable levels of ELISA antibodies due to residual maternally derived antibodies (MDA) of 5.95 and 6.69 log2 in placebo/control and vaccinated animals, respectively. The difference was not statistically significant. After vaccination, in placebo/control animals, antibody titres progressively declined, whereas a significant increase was observed in vaccinated pigs so that, starting at 2 weeks PV, the differences between the two groups were statistically

![Graph showing serologic response to PCV2 vaccination and infection](image-url)
significant. Animals in the vaccinated groups showed a continuous increase of total antibody titres, reaching a peak of ELISA antibodies at 6–9 weeks PV, with an average geometric mean ranging from 12 to 13 log₂. From this time point on, the levels of total antibodies in vaccinated groups slightly decreased even if never below a geometric mean of 6 log₂. At the last time point of blood sampling before the onset of viremia, the geometric mean of total antibodies in the placebo/control animals were under the cut-off for positivity (set at 2 log₂) in sera of pigs from farm 1 and a little over the limit (3.4 log₂) in pigs from farm 2.

In approximately 10% of the sampled population, the antibody titres at inclusion were higher than 8 log₂. In vaccinated animals these titres showed neither an increase as a consequence of vaccination nor a decline but maintained a steady course over time. In the placebo/control group, the decline of maternally derived antibodies in animals with a titre higher than 8 log₂ reached low levels within 10 weeks approximately (data not shown).

In both farms, after the onset of PCV2 viremia, seroconversion occurred within 2–3 weeks in placebo/control or vaccinated groups so that at 20–22 weeks of age the antibody levels in both groups were comparable (11–12 log₂). The geometric mean antibody titres of vaccinated animals at 26 and 35 weeks of age were lower than in placebo/control pigs because of a continuing increase of antibodies in the latter group of animals. At the last sampling of this trial at 35 weeks of age, total ELISA antibodies were above the titre of 10 log₂ in both treatment groups.

3.4.2. Serologic response to other infections

Serologic investigations performed to monitor the most frequently occurring infections in the herds (PRRSV and *M. hyopneumoniae*) found that the prevalence of PRRSV infection was 100% at 12 weeks of age in pigs from farm 1 concomitantly with *M. hyopneumoniae* seroconversion. For this latter antigen, seroprevalence continued to increase in the subsequent period.

Fig. 6. Course of individual IFN-γ PCV2-specific SC frequencies in placebo/control and PCV2-vaccinated animals after vaccination at 3 weeks of age (a = placebo/control; b = vaccinated) and after natural exposure to PCV2 (c = placebo/control; d = vaccinated), in both farms.
At farm 2, at 12 weeks of age, pigs of both groups were positive (100%) for PRRSV and still negative for M. hyopneumoniae. Seroconversion to M. hyopneumoniae started to be detected after 15 weeks of age (data not shown).

In both farms, low titres of antibodies at HI to Swine Influenza Virus were detected in some samples and were inconclusive.

3.5. Cell-mediated immune response to PCV2-vaccination and infection

PCV2-specific IFN-γ SC increased at 2 weeks after vaccination, reaching a peak 1 week later, with a mean value of 120 IFN-γ SC/10⁶ PBMC and remained at approximately the same mean value until 9 weeks of age. In the same period, at 2–3 weeks after vaccination, the number of animals with a progressively higher individual IFN-γ response increased (Fig. 6b). In unvaccinated pigs the level of PCV2-specific IFN-γ SC remained at basal levels (<20 PCV2-specific IFN-γ SC) for the entire PV period (Fig. 6a).

After the occurrence of the infection at 15–16 weeks of age, the number of PCV2-specific IFN-γ SC in vaccinated animals showed an erratic course with moderate individual increases ranging from 40 to 60 IFN-γ SC/10⁶ PBMC and remained at approximately the same mean value until 9 weeks of age. In the same period, at 2–3 weeks after vaccination, the number of animals with a progressively higher individual IFN-γ response increased (Fig. 6b). In unvaccinated pigs the level of PCV2-specific IFN-γ SC remained at basal levels (<20 PCV2-specific IFN-γ SC) for the entire PV period (Fig. 6a).

4. Discussion and conclusions

This study investigated the efficacy of a commercial PCV2a sub-type based subunit vaccine containing the capsid protein expressed in a baculovirus system under field conditions on clinical, pathologic and virologic outcomes. Aspects of the humoral and cell-mediated immune response were studied. The trial was performed in two large farms where the exposure of the animals to various pathogens such as PCV, PRRSV, M. hyopneumoniae, and commonly detected bacteria was the classical predisposing condition for PCVD (Kawashima et al., 2007). Under the conditions of this study, the onset of the clinical signs related to PCVD, in both herds, occurred during the fifth month of age and PCV2 infection was detected during the same period.

Vaccination with a single dose of the test vaccine administered intramuscularly at 3 weeks of age consistently reduced clinical signs attributed to PCVD as well as mortality and PCV2 viral load and viremia. The vaccine used for this field trial and the schedule of administration had been tested under laboratory conditions (Fort et al., 2009a); however, no peer-reviewed paper has described its efficacy under field conditions. This study sheds light on the effects of this PCV2 vaccine on farms with the presence of PCVD during the fattening period in animals aged 4-5 months, in line with the disease history in both farms. Such effects involved clinical signs and productivity (decreased numbers of pigs needing intramuscular therapy, improvement of ADWG, and decreased mortality) and PCV2 viral load in target organs of dead animals and in blood of sampled pigs in amounts and duration of viremia.

In both herds, PCV2 infection occurred in combination with other pathogens, namely, PRRSV and M. hyopneumoniae as demonstrated by serology. To measure morbidity, the number of individual treatments in unvaccinated and vaccinated animals was recorded for the duration of the study and was significantly higher in the placebo/control group. According to the statistical model applied (Poisson model) unvaccinated animals were expected to receive on average 30% more injections than vaccinated pigs. Another parameter to evaluate the efficacy of the vaccine under investigation was the comparison of ADWG. Before the onset of PCV2 viremia and associated diseases, from 3 to 12 weeks of age, no differences in ADWG were recorded. Conversely, from 12 to 26 weeks, the period when PCV2 viremia and PCVD occurred, the ADWG in vaccinated pigs was 70 g/day higher than in controls as a result of the protective effect induced by vaccination. This result is improved compared to results reported in previous studies (Fachinger et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008; Segaléš et al., 2009). The proportion of pigs that at 26 weeks of age had a body weight at least 25% lower than the mean body weight of the respective treatment group was 6.5% and 2.6% in placebo/control and vaccinated groups, respectively.

Vaccination reduced the overall mortality at both farms; the statistically significant differences were related to a decreased number of pigs suffering from PMWS and showing specific lesions in target tissues. In fact, only 1 of 408 vaccinated pigs died from PMWS. The results show that the probability of a pig vaccinated with a single dose of Porcilis® PCV at 3 weeks of age dying from PMWS was 12 times less than that of an unvaccinated control pig. Furthermore, the similar ADWG and mortality rates in both treatment groups within the first 9 weeks after vaccination indicate that the vaccine does not negatively influence the health status of the animals and suggests that the vaccine is well tolerated. All the improved parameters are associated with a significant reduction in the proportion of infected pigs and in the viral load in the blood of the animals. During the period of PCV2 infection (16–26 weeks of age on average), a high number of placebo/control pigs showed high viral loads in serum (>10⁷ DNA copies/ml), whereas in vaccinated pigs the duration of viremia and the viral load were markedly lower. In fact, in the placebo/control group, the percentages of viremic pigs with an amount of PCV2 in the blood ≥10⁶ DNA copies/ml were 70% and 42%, for farms 1 and 2, respectively. Conversely, none of the vaccinated pigs had a viral load in the blood as high as this level; it always was <10⁶ DNA copies/ml. This classification based on the amount of PCV2 load in the blood, namely <10⁶, between 10⁶ and 10⁷, and >10⁷ DNA copies/ml, is used to categorise pigs as subclinically infected, suspected, and diseased for PCV2-associated diseases, respectively (Olvera et al., 2004; Opriessnig
et al., 2007). Moreover, the classification can be used for prognostic purposes because pigs with high titres of PCV2 classified as affected with PCVD have a poor prognosis. A well-documented feature of PCV2 piglet vaccination under field conditions is the ability to diminish both the proportion of infected pigs as well as the viral load of infected animals (Fachinger et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008). The reduction of the number of PCV2-positive pigs and the viral load in vaccinated pigs is associated with the improved ADWG and reduced mortality. The approach based on qPCR is the most practical tool to monitor the efficacy of a vaccine treatment because it can be obtained from live animals (Segalés et al., 2010).

All tested animals were PCV2 negative by qPCR at the time of vaccination so that the antibody titres detected were most likely of maternal origin. However, vaccination of piglets against PCV2 with this vaccine causes a prompt seroconversion independently of the level of MDA when the titres of ELISA antibodies are below an observed seroconversion independently of the level of MDA when vaccination (ranging from 40 to 60 IFN-γ SC) already at 2 weeks after vaccination. This evidence also suggests the contribution of cell-mediated responses in vaccine-induced protection because the protective effect of PCV2 antibodies is titre-dependent and the sole induction of a humoral response might not guarantee full protection against PCV2 infection (Opriessnig et al., 2009; Fort et al., 2009b).

After the onset of infection, control pigs showed a marked increase of IFN-γ SC and at the end of the observation period these high levels of cellular response were associated with a reduction of viremia. This demonstrates that the development of adaptive immunity over the course of the spontaneous PCV2 infection in the field also involves cell-mediated responses. In accordance with the results reported by Fort et al. (2009b), IFN-γ SC responses are apparently related to viral replication. Thus, in pigs in which PCV2 viral load was low or absent, as occurred more frequently in vaccinated animals, the number of these cells is rather low or is maintained at residual levels as a consequence of the primary activation by vaccination (ranging from 40 to 60 IFN-γ SC on average). Conversely, controls exhibiting high levels of viremia and disease consistent with PCV2 replication had a higher frequency of IFN-γ SC concomitantly with the increase of PCV2 antibodies. Upon PCV2 natural infection, the observed high response in controls with high viral load can be explained by the fact that the IFN-γ SC response develops against both Cap and Rep proteins. The intensity of the generated response differs using either the Cap
protein or the whole PCV as an in vitro stimulus; IFN-γ responses obtained by stimulating PBMC with the whole virus are higher than with the Cap protein, suggesting that infected animals in which PCV2 is replicating might respond strongly to other viral components different from the Cap protein (Fort et al., 2009b). Under the conditions of this study, we cannot exclude that coinfections had a role in modulating the interaction of PCV2 and the adaptive immunity with effects on viral replication and load in vivo and on the onset of the clinical abnormalities.

Based on these observations it seems that the development of the PCV2-specific cell-mediated response might help avoid progression of PCV2 infection even if further studies are required to elucidate the inner mechanisms used by cell-mediated immunity to complete viral clearance and its major antigenic target proteins.

In conclusion, the present study demonstrates the beneficial effect of vaccination with a single dose of a PCV2 Cap vaccine against PCV2 under field conditions. The vaccination reduces the mortality rate, morbidity evaluated as number of individual treatments, and PCV2 viremia and viral load, and improves productive performances, namely, daily weight gain as well as carcass weight at the time of slaughter. These effects are strictly associated with virologic and clinical protection as a result of the immunogenicity of the tested vaccine measured as activation of either humoral or cellular immune response. Furthermore, the similar ADWG and mortality rates in both treatment groups within the first 9 weeks after vaccination, the absence of local reactions and the reduced number of injections in vaccinated animals as compared to placebo/controls in the same time period, indicate that the vaccine does not negatively influence the health status of the animals and suggests that the vaccine is well tolerated.

Acknowledgements

The authors thank Dr. Luca Bonati (University of Parma) for technical support on haematological analysis and Dr. Alberto Ermanno Cevidalli (Intervet/Schering-Plough Animal Health) for cooperation in sampling of the animals. Ph.D. studies of Dr. Marina Morganti are funded by a pre-doctoral grant of the University of Parma (Italy) in “Experimental and Comparative Immunology and Immunopathology”. This work was supported by a grant from the University of Parma (Italy) FIL 2007 (FILO797239) and also by Intervet/Schering Plough Animal Health (Boxmeer, The Netherlands).

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