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Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2

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ABSTRACT

The effects of a single-dose recombinant Porcine circovirus type 2 (PCV2) open reading frame 2 (ORF2) subunit vaccine were studied in a post-weaning multisystemic wasting syndrome (PMWS)-affected pig herd. A total of 1519 3-week-old piglets were allocated randomly into two treatment groups and either vaccinated against PCV2 or treated with a placebo. Study animals were followed from the time of vaccination until the end of finishing. Onset of PCV2 viraemia and clinical signs of PMWS (wasting, cough, dyspnoea, pallor and lethargy) were observed when animals were approximately 9–10 weeks old. Compared to placebo-treated animals, vaccinated animals had a significantly reduced PCV2 viral load and duration of viraemia ($p < 0.0001$). This reduction in viraemia was not affected by the level of maternal anti-PCV2 antibodies present at the time of vaccination. During the period of viraemia (10–26 weeks of age) vaccinated animals exhibited a 53% reduction in mortality rate ($p = 0.0010$), a 4.84 kg higher body weight gain ($p < 0.0001$) and a significant reduction in clinical signs ($p \leq 0.0004$). Furthermore, lung samples of vaccinated animals had a considerably reduced number of co-infections with PRRSV and *Mycoplasma hyorhinis* than lung samples of placebo-treated animals. These data indicate that vaccination against PCV2 alone protects pigs from clinical signs and co-infections associated with PMWS.

1. Introduction

In 1996 a new emerging disease termed "post-weaning multisystemic wasting syndrome" (PMWS) was described in reference to cases observed in Canada 5 years earlier [1]. Porcine circovirus type 2 (PCV2) was identified as an essential causative agent of this disease syndrome [2,3]. PMWS has subsequently been observed in virtually all regions of the world involved in intensive pig production [4]. The disease most commonly affects pigs between the ages of 5–15 weeks [2,5,6]. Clinical signs include a marked increase in the mortality rate, wasting, generalized enlargement of lymph nodes, respiratory signs, and occasionally pallor, jaundice and diarrhoea [7,8]. These clinical signs are not necessarily all seen at the same time in a PMWS-affected pig herd but it appears that the expression of clinical signs is indirectly linked to farm-specific co-pathogens that preferentially target different organ systems [9]. Epidemiological investigations have shown that porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae* (APP), *Streptococcus suis* and *Mycoplasma hyopneumoniae* are most commonly involved in this disease syndrome [6].

For the production of PMWS activation of the immune system has been shown to be the pivotal event [9]. Following infection with PCV2 the effects of the virus on the pig immune system have not been fully elucidated but it has been reported that the main target cells for PCV2 replication are the monocyte/macrophage lineage as well as other antigen presenting cells such as follicular dendritic cells [10]. Several studies have suggested that PCV2 infects dividing cells, macrophages and B lymphocytes, inducing apoptosis of the B cells that leads to the damage of lymphoid tissues resulting in extensive lymphocyte depletion [10]. In particular, PMWS-affected pigs show histiocytic infiltration and lymphocyte depletion of both follicle centres and parafollicular zones as well as lesions associated with the presence of PCV2 [10]. An increased susceptibility towards secondary infections [8,11] and a reduced immunological response towards PRRSV immunization has also been observed in PCV2 infected pigs [12]. Together, these facts have led some to suggest that PCV2 infection might cause immunosuppression [10,13].

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The classical diagnosis of PMWS on an individual animal level is based on the presence of clinical signs associated with PMWS, the presence of characteristic histopathological lesions in lymphoid tissues as well as the detection of PCV2 antigen within these lesions [14]. An alternative criterion for the diagnosis of PMWS is the viral load in serum (as detected by polymerase chain reaction) in association with the occurrence of clinical signs. Whereas sub-clinically infected pigs typically have less than 10^6 genomic equivalents of PCV2 per ml of serum, pigs suffering from PMWS show a viral load that is usually higher than 10^6 genomic equiv./ml and can reach values of up to 10^{12} genomic equiv./ml [4,15,16].

Until recently there have been no effective therapies against this multifactorial disease syndrome and therefore treatment and control of PMWS has focussed on ensuring good production practices that minimize stress, eliminate co-infections or minimize their impact [17]. Several PCV2 vaccine approaches have been tested under experimental conditions in mice and/or pigs [5,18–21]. The most successful vaccine candidates were those based on the induction of an active immune response against the open reading frame 2 (ORF2) encoded capsid protein of PCV2. This protein has been identified as the major immunogenic antigen of PCV2 inducing a protective antibody response [18,22]. The vaccine candidates generated decreased PCV2 viraemia and histopathological lesions following experimental challenge.

It was the purpose of this field study to evaluate the efficacy of a newly developed recombinant ORF2 subunit vaccine for active immunization of pigs against PCV2 (Ingelvac® CircoFLEX™, Boehringer Ingelheim Vetmedica GmbH). The study was conducted in a PMWS-affected herd in which PCV2 infection was complicated by co-infections with PRRSV and *Mycoplasma hyorhinis*. Special focus was placed on the characterization of the effects of vaccination on PCV2 viraemia and co-infections.

2. Materials and methods

2.1. Animals and housing conditions

The study was conducted in the Southern part of Germany. A total of 1519 hybrid pigs of commercial cross breeds (Landrace or Edelschwein (f) × Pietrain (m)) were obtained from 15 different breeding farms being part of a "pig-producer cooperative". The breeding farms differed in size (50–300 sows), management and health status. All sows on the breeding farms were routinely vaccinated against PPV and erysipelas. On some breeding farms sows were also vaccinated against PRRSV, *Escherichia coli* and/or *Clostridium perfringens*. Routine preventive measures of the piglets on all breeding farms included iron injection, tooth and tail cutting and castration. No other vaccination or medication protocols were being performed before the start of the study.

Following weaning at the age of 4 weeks, study animals of the different breeding farms were transferred to a nursery farm with an all-in-all-out production system. They were housed commingled in three barns containing pens designed to hold 60–120 pigs per pen. Flooring was partially perforated, heating was performed by an electric floor heating system and air was renewed via the roof of the stable. Four changes of food composition were performed during nursery phase. The feed provided to the animals was home-mixed, based on barley and soya.

At the age of approximately 12 weeks, pigs were moved to a growing to finish farm with an all-in-all-out production system. They were mixed again and housed in two stables with pens designed to hold 10–30 pigs per pen. Flooring in these stables was slatted concrete, air was renewed via the roof of the buildings and heating was performed by a special heat exchanger. Three changes of food composition were performed during finishing. The feed provided was a home-mixed ration based on barley, wheat, corn, and whey concentrate. Pigs remained at the finishing farm for 14–18 weeks.

Routine in-feed medication at the nursery site included prophylactic treatment with tetracycline hydrochloride for the first 10 days after arrival. General treatment of all study animals with enteroxid, colistin sulphate and tilmicosin was necessary when animals were 7–8 weeks old because of evidence of *S. suis* infection and diarrhoea. Furthermore, all animals were treated with amoxicillin and tylosin phosphate at the age of 12–15 weeks and with tetracycline hydrochloride at the age of 24 weeks due to the occurrence of respiratory signs. Commercially available products were used according to the data sheet recommendations provided by the marketing authorization holders.

2.2. Disease history

During the year prior to study initiation the selected herd had an average daily weight gain (ADWG) of 383–410 g during the nursery period and of 720–731 g during growing/finishing. The average mortality rate ranged between 3.5 and 4.8% during nursery and between 1.7 and 2.4% during finishing. The disease pattern of PMWS had become clinically apparent approximately 3 years prior to study initiation in November 2002 and PCV2 infection was serologically confirmed in December 2002. At the end of nursery/beginning of growing-finishing animals started to show typical signs of PMWS such as wasting, respiratory signs and a marked increase in the mortality rate with peak levels of up to 10%. Three months before study initiation, the diagnosis of PMWS was verified on the basis of clinical signs and PCV2 viraemia that both occurred when animals were approximately 9–13 weeks old. PRRSV and *M. hyorhinis* were identified in lung lavage samples of PCV2 positive animals as co-infecting pathogens.

2.3. Test articles

For active vaccination against PCV2, an inactivated subunit vaccine (Ingelvac® CircoFLEX™, Boehringer Ingelheim Vetmedica GmbH) was administered. The vaccine contained the ORF2 capsid protein of PCV2 as active component and an aqueous polymer (carbomer) as adjuvant. The ORF2 sequence was derived from a North American PCV2 isolate that was isolated from tonsil and liver samples of two pigs with signs of PMWS. The ORF2 sequence was subsequently inserted into a baculovirus expression system using an insect cell line derived from ovaries of the armyworm *Sodoptera frugiperda* (SF+ cells) as host. Animals were vaccinated with a minimum release dose (1.0 relative potency (RP) per ml) as determined by enzyme-linked immunosorbent assay (ELISA) relative to a reference preparation for which efficacy in the host animal has been demonstrated in a dose finding study.

The placebo control consisted of insect cell culture supernatant without PCV2 capsid protein but containing carbomer adjuvant.

2.4. Experimental design

The field trial was performed according to the principles of "Good Clinical Practice" (GCP) and followed a randomized, negative-controlled, double-blinded, parallel study design. Weight gain improvement of the vaccinated group compared to the placebo-treated groups was chosen as the primary parameter of study. A sample size of 699 animals in each group was calculated to have an 80% power to detect a difference in means of 1.5 kg. For compensation of possible dropouts a total of 1519 healthy pigs were included in this study.

Table 1
Descriptive data of vaccinated and placebo-treated animals at study initiation (3 weeks of age)

	Placebo	Vaccine
Number of animals	765	754
Number of litters	191	189
Number of males/females	390/375	392/362
Age (days)	25.4	25.4
Body weight (kg)	6.81	6.92
PCV2 antibody titre	1:642	1:675

At the day of inclusion pigs were equally distributed among two treatment groups with regard to initial body weight and litter assignment. In a first step, individual body weight of each piglet was recorded on the animal inclusion record from and on the back of the piglet. On the animal inclusion record from, all piglets per litter were then alternately sorted by weight using letters (A and B) as treatment codes. Once treatment group assignment was completed, the weight written on the piglets back was used to re-identify the individual piglets. The pigs were then ear tagged and vaccinated with either Ingelvac® CircoFLEX™ ($n = 754$) or placebo ($n = 765$). To guarantee blinding of the study the vaccine and the placebo were labelled with A and B, respectively. The test articles were administered as a single 1 ml dose intramuscularly in the right neck region when piglets were 25.4 ± 3.18 days (mean ± S.D.) old. Details of the study animals at the time of inclusion are shown in Table 1.

After weaning, pigs of both treatment groups were kept in mixed groups until the end of finishing in order ensure that all study animals were housed in similar conditions. All pigs received the same feed and were subjected to the same management practices. At each accommodation change, animals were newly mixed according the usual farm procedure.

The study was terminated when the first pigs were sent to slaughter at the age of 26 weeks.

2.5. Monitoring of clinical parameters and sample collection

Individual live body weight of all study animals was measured at 3, 10, 15, 20 and 26 weeks of age. Calculation of the mean ADWG was performed on the basis of the ADWG of animals being alive at the end of each weighing period.

Wasting animals were defined as animals whose body weight was at least 25% lower than the mean body weight of the respective treatment group. The number of wasting animals was calculated for each weighing day and treatment group.

All animals were monitored once weekly for clinical signs of PMWS by the investigator. Pigs were observed for a standardized amount of time (e.g. 10 min for one nursery pen with 60 pigs or two finishing pens with 30 pigs). Six clinical parameters (cough, dyspnoea, pallor, lethargy, diarrhoea, lameness) were investigated. The number of animals with at least one positive finding of the respective clinical parameter was subsequently calculated for the time periods before (3–10 weeks of age) and after the onset of PCV2 viraemia (10–26 weeks of age).

Dead animals or animals that had to be euthanized for reasons of animal welfare were recorded daily by the animal owner. Whenever possible, these animals were subjected to gross pathological examination within 24 h at a local pathology unit. Lung tissue samples were collected from each pig submitted for the detection of respiratory pathogens by polymerase chain reaction assays. In case of lesions samples were collected from the edge of these lesions.

At the day of inclusion, blood was collected from all animals in order to determine the PCV2 antibody titre. For quantification of PCV2 viral load in serum, blood samples from pre-selected 14% of study animals (110 pigs per treatment group) were collected on a weekly basis until pigs were 15 weeks old and every second week thereafter. Blood samples from 4% of pre-selected pigs (30 pigs per treatment group) obtained at 3, 8, 11, 15, 21 and 25 weeks of age were used to quantify the level of anti-PCV2 antibodies. In order to avoid bias of laboratory interpretation, the animal IDs of the blood samples were kept blinded at all times.

2.6. Determination of the PCV2 antibody titre

Quantification of the amount of anti-PCV2 antibodies in porcine serum samples was performed at bioScreen GmbH, Münster, Germany using an indirect fluorescence antibody titration (IFAT) assay. In brief, $2–6 \times 10^4$ PCV2 susceptible cells (VIDO-R1 cells [23,24]) were seeded onto a 96-well plate at $2–6 \times 10^4$cells/well, and inoculated with PCV2 virus ($10^{4.5}$ $TCID_{50}$/well) for approximately 48 h. After fixation of the cells with ethanol, serial dilutions of porcine serum samples were added to the plates in triplicate and incubated for 1 h at 37 °C allowing antibodies to bind if present in the serum samples. The plates were washed and stained for 1 h at 37 °C with a goat-anti-swine FITC-labelled antibody (Dianova, Germany, # 114-095-003), which allows antigen detection in infected cells using UV microscopy. The plates were read by an independent blinded investigator and individual wells reported as positive or negative. Serum antibody titres were calculated by the method of Reed and Muench using the highest dilution still showing specific IFAT reactivity and the number of positive wells per dilution. The method allowed the detection of antibody titres in a range 1:5 and 1:20,480.

2.7. Polymerase chain reactions

For quantification of the PCV2 viral load in serum, samples were analyzed in triplicate by a real-time polymerase chain reaction method as described previously [4]. Briefly, viral DNA was extracted from the serum samples using QIAmp 96 DNA blood kit (Quiagen) according to the manufacturer's instruction. The amplification was performed in a 25 μl reaction mixture containing 12.5 μl 2xTaqMan Universal Master Mix (AppliedBioSystems), 8.85 μl sterile, nuclease-free water, 22.5 pmol of primer PCV2-84-1256U21 (5′-GTA ACG GGA GTG GTA GGA GAA-3′), 22.5 pmol of primer PCV2-84-1319L21 (5′-GCC ACA GCC CTA ACC TAT GAC-3′), 3.75 pmol of a TaqMan probe (TaqMan-1286–1314) and 2 μl of the extracted DNA. The reaction was run in a real-time thermocycler (Applied BioSystems 7500 PCR System) with the following cycling times: 1 cycle at 50 °C for 120 s, 1 cycle at 95 °C for 120 s, 45 cycles at 95 °C for 15 s and 62 °C for 45 s (real time). PCV2 DNA quantification was achieved by comparison of the unknown sample with a standard curve derived from known amounts of PCV2 ORF plasmid DNA (10^4 to 10^{12} copies/ml, 10-fold dilution steps) and the cut-off level for a positive sample was set as 10^4 template copies per ml serum based on validation experiments.

Polymerase chain reaction assays were used as described in order to detect specific nucleic acids for PRRSV [25], *M. hyorhinis* [26], *M. hyopneumoniae* [27], *S. suis* [28], *Pasteurella multocida* [29], *A. pleuropneumoniae* [30], *Bordetella bronchiseptica* [31] and *H. parasuis* [32] in lung tissue samples. All polymerase chain reaction assays were performed by bioScreen GmbH, Münster, Germany.

2.8. Analysis of parameters of PCV2 viraemia

Parameters of viraemia studied were time of onset, end and duration of viraemia and viral load. Only animals that could consistently be followed over the course of the study were included in these calculations. The onset of viraemia was defined as the first positive sampling day in each animal, the end of viraemia as the

last positive sampling day per animal and the duration of viraemia as the number of days between the onset and end of viraemia. The mean of these parameters were subsequently calculated for each treatment group. The viral load of a blood sample was defined as the number of genomic equiv./ml of serum. The overall log viral load per animal was defined as the cumulative log viral loads of the respective animal over the course of the study. It provided information on the severity of viraemia for each individual animal combining two aspects, the length and the intensity of viraemia. The mean of this variable was subsequently calculated for each treatment group.

2.9. Statistical evaluation

The statistical unit was the individual pig. Homogeneity of the study population with regard to initial weight and age was analyzed by student *t*-test and sex distribution by χ^2-test. Means for body weight, weight gain and average daily weight gain were adjusted (least square means: LSMean) using the co-variables "treatment group", "breeding farm origin", "sex" and "initial body weight" and were compared between groups using an analysis of variance (general linear model: GLM). Wilcoxon Mann–Whitney test was used in order to assess differences with regard to parameters of viraemia and serology. Fisher's exact test was used to investigate possible differences in mortality. Statistical analyses were performed using SAS software release 8.2 (2001) (SAS, Cary, North Carolina: SAS Institute Inc.).

3. Results

3.1. PCV2 viraemia

The study farm was selected due to its ongoing history of PMWS. The profile of PCV2 viraemia during the course of the study is illustrated in Fig. 1. A sudden onset of PCV2 viraemia was observed in placebo-treated animals at the age of 9–10 weeks (Fig. 1A). Peak levels of 85% PCV2 positive animals were reached when animals were approximately 11–16 weeks old. From 17 weeks of age until the end of finishing, the proportion of PCV2 viraemic placebo-treated animals decreased without however reaching baseline levels again. High viral loads (>10^6 gE/ml of serum) were mainly observed in the early phase of viraemia when animals were approximately 10–15 weeks old. At the age of 11 weeks the proportion of placebo-treated animals with high viral loads (40%) was higher than the proportion of animals with low viral loads (10^4 to 10^6 gE/ml of serum; 37%). This ratio had changed markedly by the late phase of viraemia (17–25 weeks of age) due to a significant reduction of individual high viral loads.

Compared to the placebo-treated group the proportion of PCV2 positive animals in the vaccinated group was significantly lower ($p < 0.0001$). At the peak of viraemia not more than 35% of vaccinated animals had either high or low viral loads (Fig. 1B). Low viral loads predominated at all analyzed time points. The presented data thus indicate that upon vaccination against PCV2 a significant reduction in the percentage of animals with high and low viral loads can be achieved.

3.2. Influence of the maternally derived antibody titre on PCV2 viraemia

In order to investigate whether the presence of maternally derived antibodies against PCV2 had any influence on the efficacy of vaccination, PCV2 antibody titres were determined from all study animals at the day of vaccination. Following this, animals of each treatment group were assigned to classes of "low" (<1:100, approximately 20% of animals), "moderate" (1:100–1:1000, approximately 40% of animals) and "high" (>1:1000, approximately 40% of animals) initial antibody titres. The onset, end and duration of viraemia and the overall log viral load were subsequently calculated for animals belonging to the different PCV2 antibody titre classes.

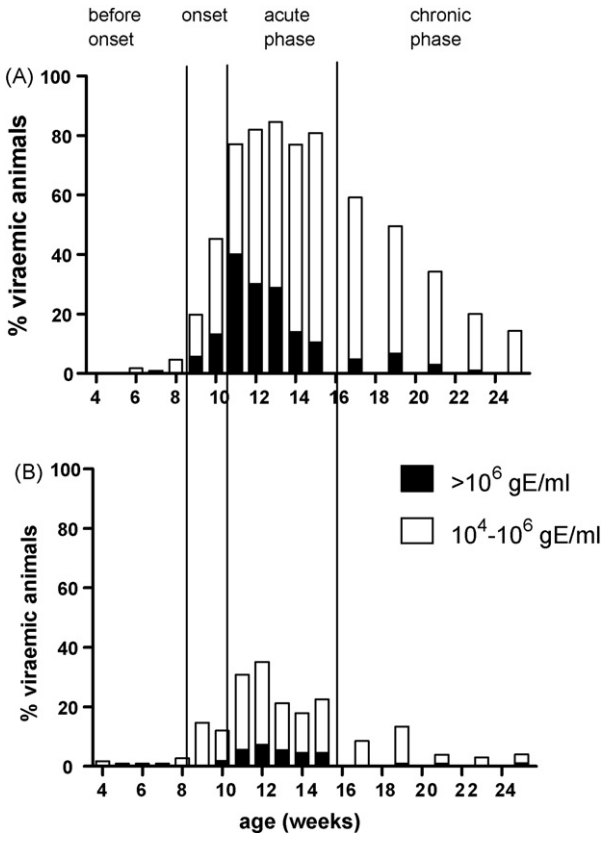

Fig. 1. Comparison of the PCV2 viral load in vaccinated and placebo-treated animals. Blood samples from pre-selected placebo-treated animals (A; $n = 110$) and vaccinated animals (B; $n = 110$) were collected until 15 weeks of age and every second week thereafter. On the basis of the quantitative PCR results animals were grouped into classes of animals with sub-clinical viral loads (10^4 to 10^6 gE/ml) and clinical relevant viral loads (>10^6 gE/ml).

As indicated in Table 2, the mean onset of PCV2 viraemia for placebo-treated animals was observed 47 days after vaccination when animals were approximately 10 weeks old. The mean end of PCV2 viraemia in placebo-treated animals was observed 108 days after vaccination when animals were approximately 18 weeks old, resulting in a mean duration of viraemia of 60 days. Placebo-treated animals with high initial PCV2 antibody titres had a later onset and a shorter duration of viraemia as well as a lower overall log viral load than placebo-treated animals with low initial PCV2 antibody titres.

Vaccinated animals had a comparable onset of PCV2 viraemia as placebo-treated animals but a significantly earlier end of PCV2 viraemia ($p \leq 0.0001$) resulting in an approximately 30 days shorter duration of viraemia ($p \leq 0.0001$). Similarly, the overall $\log_{10}$ viral load was approximately 70% lower than in placebo-treated animals ($p \leq 0.0001$). These statistical significant differences in the end and duration of viraemia and in the viral load were observed for animals of each of the three PCV2 antibody titre classes ($p \leq 0.0205$). Furthermore, vaccinated animals with high initial PCV2 antibody titres appeared to have a later onset of viraemia than vaccinated animals with low initial PCV2 antibody titres. However, there were no considerable differences in duration of viraemia and the viral load between vaccinated pigs with low and high initial PCV2 antibody titres.

Table 2
Parameters of viraemia in correlation with PCV2 antibody titre present at the time of vaccination

		Titre score[a]							
		Total		<1:100		1:100–1:1000		>1:1000	
		Placebo, 89[b]	Vaccine, 66[b]	Placebo, 18[b]	Vaccine, 11[b]	Placebo, 42[b]	Vaccine, 31[b]	Placebo, 29[b]	Vaccine, 24[b]
Onset (days)	Mean	47.2	50.7	43.8	34.6	45.5	54.9	51.9	52.7
	p-value	0.0863		0.0965		0.0046		0.7030	
End (days)	Mean	108.0	80.8	112.6	71.2	105.8	81.8	108.2	83.8
	p-value	<0.0001		<0.0001		<0.0001		<0.0001	
Duration (days)	Mean	60.7	30.0	68.8	36.5	60.3	26.9	56.3	31.1
	p-value	<0.0001		0.0205		<0.0001		0.0066	
Viral load[c] (gE/ml)	Mean	33.9	9.6	38.2	10.8	35.6	9.7	28.9	8.9
	p-value	<0.0001		<0.0001		<0.0001		<0.0001	

[a] PCV2 antibody titre at the time of vaccination.
[b] Treatment, number of animals.
[c] Viral load indicates the mean overall $\log_{10}$ viral load derived from the sum of genomic equiv.(gE)/ml per animal of 17 samplings days.

Taken together, the presented data indicate that the PCV2 vaccine is capable to reduce PCV2 viraemia irrespective of the maternal antibody status.

3.3. Serological response to vaccination

Before vaccination antibody titres of both treatment groups against PCV2 were moderate with a mean geometric titre in placebo-treated animals of 1:642 and in vaccinated animals of 1:675 (Fig. 2). At 7 weeks of age the mean antibody titre in placebo-treated animals had fallen to a level of 1:247. The onset of PCV2 viraemia occurred at 9–10 weeks of age (compare Fig. 1) and was followed by seroconversion at approximately 11 weeks of age. Consistently high serum antibody titres (Geometric mean titre (GMT): ≥1:15,208) were reached from 15 weeks of age onwards. Vaccinated animals had a significantly higher geometric mean PCV2 antibody titre 4 weeks after vaccination ($p = 0.0033$) and a considerably higher geometric mean PCV2 antibody titre at the time of seroconversion (11 weeks of age) compared to placebo-treated animals. Interestingly, geometric mean antibody titres of vaccinated animals at 21 and 25 weeks of age were lower than in placebo-treated animals (GMT: ≤1:14,092 in vaccinated animals compared to ≤1:19,946 in placebo-treated animals).

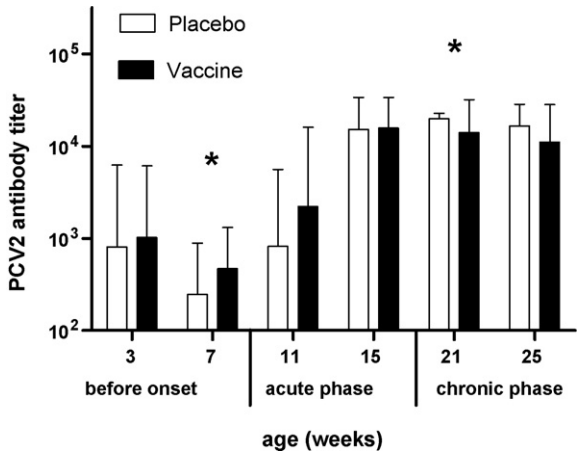

Fig. 2. Comparison of the mean PCV2 antibody titres. The PCV2 antibody titres were determined in all vaccinated animals ($n = 751$) and placebo-treated animals ($n = 764$) at study initiation and in always the same 4% of vaccinated ($n = 30$) and placebo-treated animals ($n = 30$) at the age of 7, 11, 15, 21 and 25 weeks. Log_{10} transformed mean antibody titres are shown for placebo-treated animals and vaccinated animals. Standard deviation is indicated by error bars. Asterisks present significance levels of $p < 0.05$.

3.4. Comparison of the average daily weight gain during the course of the study

Average daily weight gain was considered to be an objective measurable parameter to determine the severity of PMWS and the effects of vaccination in a large number of animals. Table 3 shows the ADWG (Least Square Mean) in vaccinated and placebo-treated animals for the intervals between five different weighing time points as well as for the finishing period (10–26 weeks of age) and the entire time of study conduction (3–26 weeks of age). No differences in the ADWG between the treatment groups were observed during the period before onset of viraemia when animals were 3–10 weeks old ($p = 0.9062$). In the subsequent time period (10–15 weeks of age) vaccinated animals had an 82 g/day higher weight gain than placebo-treated animals ($p < 0.0001$). Between 15 and 20 weeks of age the ADWG was still

Table 3
Average daily weight gain (g/day) during different study intervals

Growth period	Age (weeks)	Placebo[a] (95% CI)	Vaccine[b] (95%CI)	Difference[c]	*p*-value
Daily weight gain (g/day)					
Nursery	3–10	358 (353–364)	358 (353–363)	0	0.9062
Beginning of finishing	10–15	576 (564–589)	658 (646–671)	82	<0.0001
Middle of finishing	15–20	790 (777–803)	836 (823–849)	46	<0.0001
End of finishing	20–26	774 (760–787)	799 (786–813)	26	0.0024
Entire finishing	10–26	722 (714–731)	766 (758–775)	44	<0.0001
Total	3–26	619 (613–626)	650 (644–656)	30	<0.0001

[a] $n = 744, 729, 708, 657, 656, 657$ animals for 3–10, 10–15, 15–20, 20–26, 10–26 and 3–26 weeks of age.
[b] $n = 731, 722, 718, 680, 680, 680$ animals for 3–10, 10–15, 15–20, 20–26, 10–26 and 3–26 weeks of age.
[c] Values of the vaccinated group minus the placebo-treated group.

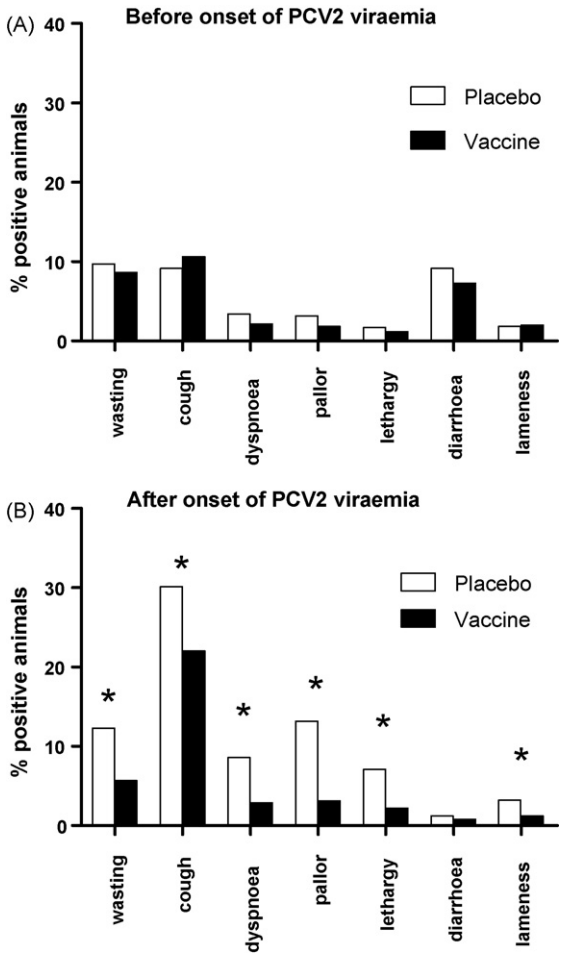

Fig. 3. Clinical signs after the onset of PCV2 viraemia. All animals were monitored weekly for signs of cough, dyspnoea, pallor, lethargy, diarrhoea and lameness. The percentage of animals with apparent clinical findings was calculated for all placebo-treated animals and vaccinated animals for the time from 3–10 weeks of age (A) and 10–26 weeks of age (B). Wasting animals were defined as the number of animals with an ADWG 25% lower than the mean of the respective treatment group at 10 weeks of age (A) and 15 weeks of age (B). Asterisks present significant levels of $p < 0.05$.

46 g/day higher in vaccinated animals than in placebo-treated animals ($p < 0.0001$). During the final finishing when animals were between 20 and 26 weeks old the ADWG of vaccinated animals was 26 g/day higher ($p = 0.0024$). Together this resulted in a 4.84 kg (44 g/day) higher weight gain during the finishing period and a 4.70 kg (30 g/day) higher weight gain during the entire study period.

3.5. Analysis of clinical signs in vaccinated and placebo-treated animals

Clinical signs were monitored weekly in all animals and animals with apparent clinical findings were recorded. Before onset of viraemia (3–10 weeks of age) the frequency of clinical signs was generally low with no significant differences between the treatment groups ($p \geq 0.1587$; Fig. 3A). After the onset of PCV2 viraemia (10–26 weeks of age) coughing was the predominant clinical sign among both groups (Fig. 3B). A considerable proportion of placebo-treated animals also showed signs of wasting, pallor and lethargy while signs of diarrhoea and lameness were less prominent. Compared to placebo-treated animals a significantly lower number of vaccinated animals showed signs of wasting ($p < 0.0001$), cough

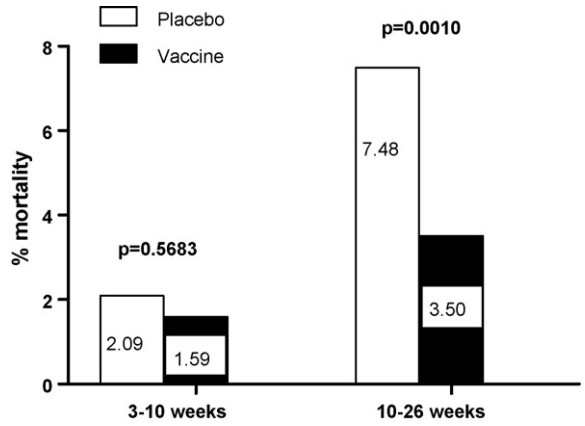

Fig. 4. Analysis of the mortality rate. The mortality rate (%) of vaccinated pigs and placebo-treated pigs was compared for the time periods before onset of PCV2 viraemia (3–10 weeks of age) and after onset of PCV2 viraemia (10–26 weeks of age).

($p = 0.0004$), dyspnoea ($p < 0.0001$), pallor ($p < 0.0001$) and lethargy ($p < 0.0001$).

3.6. Reduction of the mortality in vaccinated animals

As indicated in Fig. 4, the mortality rates in both groups were comparable before onset of viraemia (3–10 weeks of age). During the period following the onset of viraemia (10–26 weeks of age) placebo-treated animals had a 53% higher mortality rate than vaccinated animals (7.48% versus 3.50%, $p = 0.0010$). Wasting (30%) and pneumonia (21%) were the two most frequent diagnosed main clinical entities recognised at *postmortem* examination in placebo-treated animals after the onset of viraemia. Among the few vaccinated animals that died or had to be euthanized after the onset of viraemia, the reasons for death or removal from the study were more varied and included fractures, intestinal torsions, pneumonia, enteritis, exophthalmus and sudden heart failure (Table 4). Whenever possible, animals with unknown or infectious causes of death (approximately 40% of dead animals of both treatment groups) were transferred to the local pathology unit. Upon necropsy of these animals, a poor body condition, enlarged lymph nodes and severe pneumonia were the most often reported findings.

Together, the presented data give evidence for a significant PMWS related increase in the mortality rate in placebo-treated animals after the onset of PCV2 viraemia.

3.7. Reduction of co-infections by vaccination against PCV2

To obtain further information about concurrent, respiratory infections in the animal herd microbiological analyses were per-

Table 4
Main clinical entity recognized prior death or euthanasia in 10–26 week old animals

	Placebo		Vaccine	
	%	(*N*)	%	(*N*)
Wasting	30	(17/56)	4	(1/26)
PDNS	4	(2/56)	0	(0/26)
Pneumonia	21	(12/56)	19	(5/26)
Enteritis	9	(5/56)	15	(4/26)
Lameness	5	(3/56)	8	(2/26)
CNS disturbance	7	(4/56)	0	(0/26)
Others[a]	23	(13/56)	54	(14/26)

[a] Other reasons for death or removal from study were fractures, intestinal torsions or remained unknown.

Table 5
Percentage (number) of positive findings in lungs of dead animals after the onset of viraemia (10–26 weeks of age)

	Placebo		Vaccine		*p*-Value
	%	(*N*)[a]	%	(*N*)[a]	
PCV2	92	(24/26)	55	(6/11)	0.0158[b]
PRRSV	50	(13/26)	27	(3/11)	0.2847
M. hyorhinis	62	(16/26)	18	(2/11)	0.0293[b]
M. hyopneumoniae	23	(6/26)	45	(5/11)	0.2436
S. suis	12	(3/26)	27	(3/11)	0.3351
P. multocida	4	(1/26)	9	(1/11)	0.5120
APP	8	(2/26)	0	(0/11)	1.0000
B. bronchiseptica	0	(0/26)	0	(0/11)	–
H. parasuis	0	(0/26)	0	(0/11)	–

[a] Number of positive lungs/total lungs analyzed by polymerase chain reaction (PCR).
[b] Statistically significant ($p < 0.05$).

formed on lung samples of animals that were sent for pathological examination. Comparison of the frequency of pathogens detected in lung samples revealed no major differences among both treatment groups for the time before onset of PCV2 viraemia (data not shown). After the onset of PCV2 viraemia (10–26 weeks of age) the percentages of lung samples which were tested positive for *M. hyorhinis* and PRRSV were 71% ($p = 0.0293$) and 46% ($p = 0.2847$), respectively lower in vaccinated animals compared to placebo-treated animals (Table 5). No significant differences in the number of lung samples positive for *M. hyopneumoniae*, *S. suis* or *P. multocida* were observed ($p \geq 0.3351$) between the treatment groups. These pathogens were either present at only low frequencies (*S. suis*, *P. multocida*) or only appeared at a very late phase of PCV2 infection (20 weeks of age; *M. hyopneumoniae*) when the majority of animals had relatively low PCV2 viral loads.

As shown in Fig. 5, combinations of two to three pathogens were detected in a large number of single lung samples. In placebo-treated animals, triple infections with PCV2, PRRSV and *M. hyorhinis* were most commonly observed. Compared to placebo-treated animals the detection rate of triple infections in vaccinated animals were considerably lower and dual infections with PCV2 and *M. hyorhinis* were not detected.

These data suggest that in the selected animal herd vaccination against PCV2 is capable of reducing the number of PCV2 co-infections.

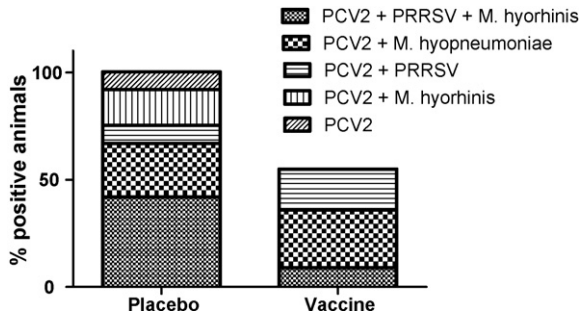

Fig. 5. Combinations of co-infections after the onset of PCV2 viraemia. Lung samples collected from 26 placebo-treated and 11 vaccinated animals after the onset of viraemia (10–26 weeks of age) were analyzed by PCR in order to detect specific nucleic acids for PCV2, PRRSV and *Mycoplasma hyorhinis*. The relative percentage of the different combinations of these pathogens was calculated for placebo-treated and vaccinated animals on the basis of the total number of analyzed lung samples per treatment group.

4. Discussion

PMWS was first described in 1996, referring to cases observed in Canada in 1991. Since then, PMWS has been recognized worldwide and is considered to be a serious threat to the swine industry. Recently a vaccine for active immunization of pigs against PCV2 has been developed containing a baculovirus expressed ORF2 capsid protein as active substance and an aqueous polymer (carbomer) as adjuvant (Ingelvac® CircoFLEX™). This study aimed to investigate the efficacy of this PCV2 vaccine under field conditions in helping to control a PCV2 related multifactorial disease syndrome such as PMWS.

For the conduction of the study a "pig producer cooperative" was selected in which piglets from 15 different breeding farms were mixed in large pens on one nursery farm and later transferred to one finishing farm. The exposure of the herd to various pathogens such PCV2, PRRSV, *M. hyopneumoniae* and *M. hyorhinis* and the stress of "crowding" were considered to be classical predisposing factors of PMWS [11]. Since the first diagnosis of PMWS 3 years before study initiation, the selected farm system had never been able to overcome this disease complex and onset of clinical signs was consistently reported to occur at the end of rearing/beginning of finishing.

In line with this disease history, onset of PCV2 infection was observed in this study when animals were approximately 9–10 weeks old. On a herd level the time of viraemia could be differentiated into an acute and a chronic phase. Especially during the acute phase of PCV2 viraemia (11–16 weeks of age) a high number of placebo-treated animals showed high viral loads ($>10^6$ genomic equiv./ml) in serum. During the chronic stage of infection (17–26 weeks of age) the mean PCV2 viral load was decreasing. Under the influence of vaccination the duration of viraemia and the viral load were significantly lower.

Interestingly, the reduction in the number of PCV2 positive animals and in the viral load seemed to be sufficient to significantly improve body weight gain and reduce mortality in the vaccinated group. The highest differences in weight gain between vaccinated and placebo-treated animals were observed during the acute phase of infection while at the late phase of infection the poorer growth in placebo-treated animals was less pronounced. This is in line with other field and laboratory studies in which a correlation between a viral load of $>10^6$ PCV2 genomic equiv./ml and the severity of clinical PMWS observations has also been reported [4,15,16].

The predominant clinical signs observed during this study were respiratory signs. In general the clinical signs of PMWS have been reported to be indirectly linked to farm-specific co-pathogens that preferentially target different organ systems [9]. The presence of PRRSV, *M. hyorhinis* and *M. hyopneumoniae* may therefore explain the high percentage of placebo-treated animals with respiratory signs. While the potential involvement of PRRSV in PMWS has often been described [3,6,11,33,34] the role of *M. hyorhinis* in this disease complex is not that clear since *M. hyorhinis* is considered as part of the normal flora of the upper respiratory tract [35]. However, contribution to pneumonia as a secondary pathogen has also been reported [36] and in particular a relationship between PRRSV and *M. hyorhinis* has been previously observed with more severe pulmonary lung lesions in dually infected animals than in animals infected with either pathogen alone [11]. Furthermore an association between PCV2, PRRSV and *M. hyorhinis* has been found in a recent epidemiological study in Japan. It was demonstrated that the frequency of co-infections with PRRSV and *M. hyorhinis* in PMWS-affected animals was significantly higher than in PMWS-negative animals [11]. In our study combinations of up to three respiratory pathogens were detected in a single lung sample supporting the

multifactorial disease character of PMWS in the selected animal herd.

Under the influence of vaccination the percentage of animals with signs of wasting, cough and dyspnoea was significantly lower. This implies that vaccination against PCV2 might also have an impact on the prevalence of other co-pathogens. In our study considerably more lung samples from placebo-treated animals than from vaccinated animals examined after the onset of PCV2 viraemia were found to be positive for *M. hyorhinis* and PRRSV. Furthermore the number of co-infecting pathogens found in single lung samples was higher in placebo-treated animals than in vaccinated animals. These findings suggest that PCV2 infection makes animals more susceptible to co-infections with PRRSV and *M. hyorhinis* and therefore supports the theory of a PCV2 mediated immunosuppression in PMWS-affected animals [10,13]. Furthermore it indicates that vaccination against PCV2 alone does also lower the incidence of co-infecting agents such as PRRSV and *M. hyorhinis* in PMWS-affected animals.

The beneficial effects of vaccination were independent of the level of maternally derived antibodies present at the time of vaccination. This was demonstrated by comparing parameters of viraemia in groups of animals with low (<1:100), moderate (1:100–1:1000) and high (>1:1000) PCV2 antibody titres. The fact that all tested animals were PCV2 negative by qPCR at the time of vaccination suggests that animals had not been exposed to PCV2 until the age of 3 weeks and that the antibody titres present at the time of vaccination were most likely of maternal origin. The thresholds used to classify maternally derived antibody (MDA) titres into the three titre classes are consistent with those of a Canadian field study in which MDA titres of <1:80, 1:640, and >1:1280 were considered as low, moderate and high, respectively [37]. Furthermore, in a Spanish field study a correlation between the antibody titre and protection from disease has been demonstrated. Pigs with low antibody titres at 7 weeks of age (mean antibody titre 1:100, range 0 to 1:320) had a significantly higher mortality rate over the following 5 weeks than animals with higher antibody titres [38]. This suggests that PCV2 maternal antibody titres below 1:100 cannot be regarded as being maximally protective against PCV2 infection. The fact that placebo-treated animals with low maternally derived antibodies had a slightly earlier onset, a longer duration and a higher viral load than placebo-treated animals with moderate or high maternal antibodies is in line with these findings and therefore justifies the grouping of animals into these three antibody classes.

In case of possible colostral antibody interference it was expected that the vaccine would be less effective in animals with "high" maternally derived antibody titres resulting in a longer duration of viraemia and a higher viral load than in animals with "low" maternally derived antibody titres. However, it could be shown that vaccinated "high titre" animals had a duration of viraemia and viral loads comparable with vaccinated "low titre" animals and that viraemia in vaccinated "high titre" animals was significantly reduced compared to placebo-treated "high titre" animals. Accordingly, the use of a killed baculovirus-expressed PCV2 ORF2 vaccine with a sufficiently high antigen content and carbomer as adjuvant appears to be suitable for active immunization of seropositive piglets against PCV2.

The significant increase in the antibody titre of vaccinated animals compared to placebo-treated animals 4 weeks following vaccination could be regarded as one possible immune effector mechanism which is induced after the administration of the vaccine. However, the presence of maternal antibodies in all animals around the time of vaccination does not allow the use of this increase in the antibody titre as a reliable tool to differentiate between vaccinated and placebo-treated animals. Higher antibody titres were also observed in vaccinated animals at the time of seroconversion (11 weeks of age) which suggests a quicker or more intense humoral immune response towards PCV2 during the acute phase of infection. Interestingly, higher antibody titres in placebo-treated animals compared to vaccinated animals were found during the chronic phase of PCV2 infection (21 and 25 weeks of age). This finding could potentially be the result of a lower level of viraemia in vaccinated animals avoiding an extensive PCV2 antibody production in these animals.

Finally, the similar ADWG among both treatment groups as well as the slightly lower mortality rate in vaccinated animals within the first 7 weeks following vaccination indicates that the vaccine does not negatively influence the general health status of the animals within the first weeks after vaccination. These findings therefore suggest that the vaccine is well tolerated.

Taken together our study was suitable to demonstrate the beneficial effects of vaccination against PCV2 in a PMWS-affected herd. Under the influence of a single-dose recombinant PCV2 vaccine, a lower rate of mortality and clinical signs, an improved weight gain as well as a reduction in viraemia and in the number of co-infections with PRRSV and *M. hyorhinis* in lung samples were detected. The study thus demonstrates that vaccination against PCV2 alone is effective in reducing signs of PMWS under normal pig husbandry conditions.

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