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Efficacy and safety of a combined Porcine Circovirus and *Mycoplasma hyopneumoniae* vaccine in finishing pigs

Maarten Witvliet *, Hans Holtslag, Tom Nell, Ruud Segers, Vicky Fachinger

MSD Animal Health, PO Box 31, 5830 AA Boxmeer, The Netherlands

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ABSTRACT

The safety and protective efficacy of a new one dose combination vaccine containing Porcine Circovirus type 2 (PCV2) and *M. hyopneumoniae* antigens – Porcilis® PCV M Hyo - was evaluated in laboratory studies and under field conditions. Vaccination resulted in a moderate temperature increase on the day of vaccination and mild systemic and local reactions were found in only a low percentage of the vaccinated pigs. The local reactions observed were small (max. 2 cm) and transient (max. 1 day). In short term (onset of immunity) and long term (duration of immunity) challenge studies with the individual pathogens, the vaccine significantly reduced the PCV2 load in lymphoid tissue and lungs and *M. hyopneumoniae*-induced lung lesions. In a placebo-controlled field trial on a farm where both PCV2 and *M. hyopneumoniae* were present, vaccination of piglets at 3 weeks of age resulted in a reduction of PCV2 viremia and shedding and lower lung lesion scores at slaughter. In addition, a positive effect on the average daily weight gain (+ 34 g/day) in the finishing phase was observed. It can therefore be concluded that this new ready to use combination vaccine is safe and efficacious against PCV2 and *M. hyopneumoniae* single and combined infections.

1. Introduction

Porcine Circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* are the two most prevalent pathogens encountered in today's pig industry. PCV2 was originally identified as the causative agent of the "Postweaning Multisystemic Wasting Syndrome", but is also involved in a number of other disease syndromes which have been collectively named Porcine Circovirus Diseases (PCVD) [1,2]. The most pronounced PCVDs are Porcine Respiratory Disease Complex (PRDC), Porcine Dermatopathy and Nephropathy Syndrome, reproductive failure, granulomatous enteritis, congenital tremors and exudative epidermitis. Subclinical PCV2 infections are characterized by poor growth performance in apparently healthy pigs [3–5]. Considering that up to 100% of pigs are seropositive for PCV2 at the time of slaughter, subclinical PCV2 infection is currently considered to be the major form of PCVD [4,5].

M. hyopneumoniae is a respiratory pathogen in pigs and is the primary causative agent of enzootic pneumonia (EP), a chronic disease in pig herds [6,7]. *M. hyopneumoniae* in association with other bacterial and viral agents is also implicated in the PRDC. EP and PRDC cause important economic losses to the swine industry due to reduced performance (growth rate, feed conversion ratio) and increased antibiotic use [7].

Vaccines against PCV2 [8,9] and *M. hyopneumoniae* [10,11] are routinely used in the pig industry, and it has been shown that concurrent vaccination with PCV2 and *M. hyopneumoniae* vaccines can provide protection against both pathogens under laboratory conditions [12]. However, for the convenience of the user and to reduce the number of injections given to piglets, a ready-to-use combination product, preferably given as a one dose regimen, would be highly desirable. Therefore, the objective of the present studies was to evaluate the efficacy and safety of a new ready-to-use combination product based on the *M. hyopneumoniae* monovalent vaccine M+PAC® (MSD Animal Health) and the PCV vaccine Porcilis® PCV (MSD Animal Health) under laboratory and field conditions.

2. Materials and methods

2.1. Vaccine

A vaccine containing inactivated *M. hyopneumoniae* cells, baculovirus-expressed ORF2 antigen of PCV2 and the Emunade® adjuvant (Porcilis® PCV M Hyo, MSD Animal Health) was tested. Emunade® is a combination of an oil-in-water emulsion with aluminium hydroxide. The vaccine was given intramuscularly as a single 2 ml dose to 3 week old piglets according to the product leaflet.

* Corresponding author.
E-mail address: maarten.witvliet@merck.com (M. Witvliet).

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2.2. Safety studies

2.2.1. Laboratory study

Two groups of 12 healthy SPF pigs were either vaccinated with Porcilis® PCV M Hyo at 19–21 days of age (vaccine group) or injected with phosphate buffered saline (control group). Until 14 days post vaccination (dpv), the piglets were observed daily for abnormal systemic and local reactions. Rectal temperature was recorded one day before vaccination, just before vaccination, 4 h after vaccination and daily for four days. At 14 dpv, all animals were sacrificed for examination of the injection site.

2.2.2. Field study

A GCP field safety study was done in young piglets according to a randomized and blinded design in two pig farms in The Netherlands and one in Germany. In each farm, at least 56 healthy three-week-old suckling piglets aged 17–24 days were allocated randomly to one of two groups. The piglets in one group (vaccine) were vaccinated with Porcilis® PCV M Hyo and the piglets in the other group (control) were injected with sterile buffered saline. The general health of the piglets was checked at admission (one day before vaccination), just before vaccination, 1 and 4 h after vaccination and daily for 14 days. One day before vaccination, just before vaccination, 4 h after vaccination and daily for 4 days after vaccination, the rectal temperature of all piglets was measured. The injection site was examined for local reactions by palpation at 1 and 4 h after vaccination and then daily for 14 days. All study piglets were weighed individually at admission (day-1) and at the end of the study 3 weeks post vaccination (wpv).

2.3. Efficacy studies

2.3.1. Laboratory studies

The onset of immunity (OOI) and duration of immunity (DOI) for each of the two vaccine antigens were determined in experimental challenge studies (Table 1). In each experiment, 3 week old pigs from herds free of *M. hyopneumoniae* and seropositive for PCV2 were randomly divided in two groups (vaccine and control) at the time of vaccination. Blood samples were taken just before vaccination, at the time of challenge and 2 (PCV2 challenge studies only) and 3 weeks after challenge. In the DOI studies, blood samples were also taken at regular intervals between vaccination and challenge.

PCV2 challenge was done by intranasal instillation (3 ml per nostril, $\pm 10^6$ $TCID_{50}$) of a recent Dutch field isolate at 2 wpv or 22 wpv. Three weeks after PCV2 challenge, all pigs were necropsied and the mesenteric and inguinal lymph nodes, tonsil and lung were collected for quantification of the PCV2 viral load.

M. hyopneumoniae challenge was performed intratracheally on two consecutive days with 10 ml of a culture of a Danish field isolate (provided by Dr N. Friis, National Veterinary Laboratory, Copenhagen) containing $\pm 10^7$ CCU/ml at 4 wpv or 21 wpv. Three weeks after challenge, the pigs were necropsied to evaluate lung lesions which were scored as described [13]; the maximum score is 55.

During the studies, pigs were observed daily for clinical abnormalities.

Table 1
Overview of the laboratory challenge studies. Piglets were vaccinated at three weeks of age.

Type of study	Group	No. of piglets	Challenge at (weeks post vaccination)	Challenge
Onset of immunity	Porcilis PCV M Hyo	15	2	PCV2
	Control	15		
Onset of immunity	Porcilis PCV M Hyo	19	4	*M. hyopneumoniae*
	Control	19		
Duration of immunity	Porcilis PCV M Hyo	15	22	PCV2
	Control	15		
Duration of Immunity	Porcilis PCV M Hyo	40	21	*M. hyopneumoniae*
	Control	40		

2.3.2. Field study

A GCP combined field safety and efficacy study was performed according to a controlled, randomized and blinded design in a French pig herd with a *M. hyopneumoniae* and a PCV2 infection. Healthy three week old suckling piglets were allocated randomly, within litters, to one of two groups of approximately 300 piglets each. The pigs in one group (vaccine) were vaccinated with Porcilis® PCV M Hyo and the pigs in the other group (control) were injected with sterile buffered saline. The pigs were weighed individually at vaccination, at transfer to the finishing unit and before slaughter. Medication was recorded and pigs that died during the study were examined post mortem to establish the cause of death. The lungs were examined individually at slaughter to score the severity of typical *M. hyopneumoniae* lesions and pleurisy. Twenty five piglets per treatment group were bled for serum samples and rectal and nasal swabs were taken approximately every 4 weeks. Although safety was not the primary objective of this study, the investigator routinely observed the animals at vaccination and, as a group, at 4 h after and 1, 4, 7 and 14 days after vaccination. The primary efficacy parameters were *M. hyopneumoniae*-like lung lesions at slaughter, PCV2 viral load in serum (PCV2 viremia) and the average daily weight gain (ADWG) during finishing, (i.e. between 7 and 19 wpv). Secondary parameters were overall ADWG (i.e. between vaccination and 19 wpv), mortality, morbidity (individual medication), pleurisy lesions and PCV2 shedding. Also the serological response to vaccination or field infection was determined.

2.4. Serology

For *M. hyopneumoniae*, a commercial ELISA (IDEXX, M. hyo Ab test) was used according to the manufacturer's instructions. Results were expressed as negative, positive or inconclusive according to the product leaflet. For PCV2, an in-house ELISA was performed as previously described [14].

2.5. Quantification of PCV2 DNA

Quantification of the PCV2 viral load in serum, lymphoid organs, lung and excretions was performed by qPCR as previously described [14]. In brief, viral DNA was extracted using DNA/Viral NA SV 1.0 kit. The amplification was performed in a reaction mixture containing 10 µl extracted DNA, 1.5 µl (15 mM) of forward primer (5′-TggCCCgCAgTATTTCTgATT-3′), 1.5 µl (15 mM) of reverse primer) (5′-ggggAAAgggTgACgAACTg-3′), 2.0 µl (20 mM) DLHP probe (5′-FAM-CCAgCAATCA-gACCCCgTTggAATg-TAMRA-3′), 5.0 µl dNTPs (SphaeroQ), 1.0 µl SuperTaq (SphaeroQ) and 29 µl PCR buffer. The reactions were performed in a real-time thermocycler with the following cycling times: 1 cycle at 50 °C for 120 s, 1 cycle at 95 °C for 600 s, 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. To allow comparison of the viral load of different sample types (serum, tissues, swabs) results of the viral load are expressed as copies per µl DNA extract. During validation of the PCR, the limit

of quantification was found to be 1.6 template copies per µl DNA extract.

2.6. Statistical analyses

In the laboratory safety study, the rectal temperatures after vaccination were compared in a 2-sample *t*-test. Rectal temperatures in the field safety study were analyzed using a repeated measures ANOVA model with farm and interaction farm × treatment group as random effects, treatment group as fixed effect and mean pre-vaccination temperature as covariate.

Lung lesion scores in challenge experiments and the qPCR data of inguinal and mesenteric lymph nodes, lung and tonsil were analyzed by the Wilcoxon Rank Sum test. PCV2 serology data at 2 wpv (OOI study) were analyzed by ANOVA and the serology in the time period 0–22 wpv (DOI study) was analyzed by ANOVA for repeated measurements. The PCV2 serology in the field study was also analyzed by repeated measures ANOVA.

For the serum, nasal and fecal samples collected after the onset of the PCV2 infection in the field study, the areas under the curve (AUC) of the qPCR data were calculated by the linear trapezoidal rule and ranked before analysis using ANOVA with vaccination group, production batch and their interaction as fixed effects. Lung lesion scores in the field study were compared between the groups using mixed model ANOVA. Vaccination group, production batch and their interaction were included as fixed effects and the

Table 2
Analysis of the safety of the PCV-*M. hyopneumoniae* combination vaccine.

		Vaccine	Control	p-values
Laboratory safety study	Number of pigs (n)	12	12	
	Pigs with local reactions (%)	0	0	1.000
	Pigs with macroscopically visible local reactions at necropsy (%)	0	0	1.000
	Pigs with a systemic reaction (%)	0	0	1.000
	Rectal temperature at 4 h post vaccination (°C)	40.5 ± 0.4	39.4 ± 0.2	<0.001
Field safety study	Number of pigs (n)	84	85	
	Pigs with local reactions (%)	13.1	3.5	0.0276
	Pigs with a systemic reaction (%)	6.0	4.7	0.7464
	Rectal temperature at 4 h post vaccination (°C)	40.6 ± 0.6	39.5 ± 0.4	<0.0001
	average daily weight gain (g/day) during the observation period(0–3 wk post vaccination)	245 ± 8	248 ± 8	0.7053
Field safety and efficacy study	Number of pigs (n)	302	303	
	Pigs with local reactions (%)	1.3	0.7	0.4504
	Pigs with a systemic reaction (%)	2.6	0.7	0.0630
	Average daily weight gain (g/day) during nursery (0–7 wk post vaccination)	360 ± 4	369 ± 5	0.0839

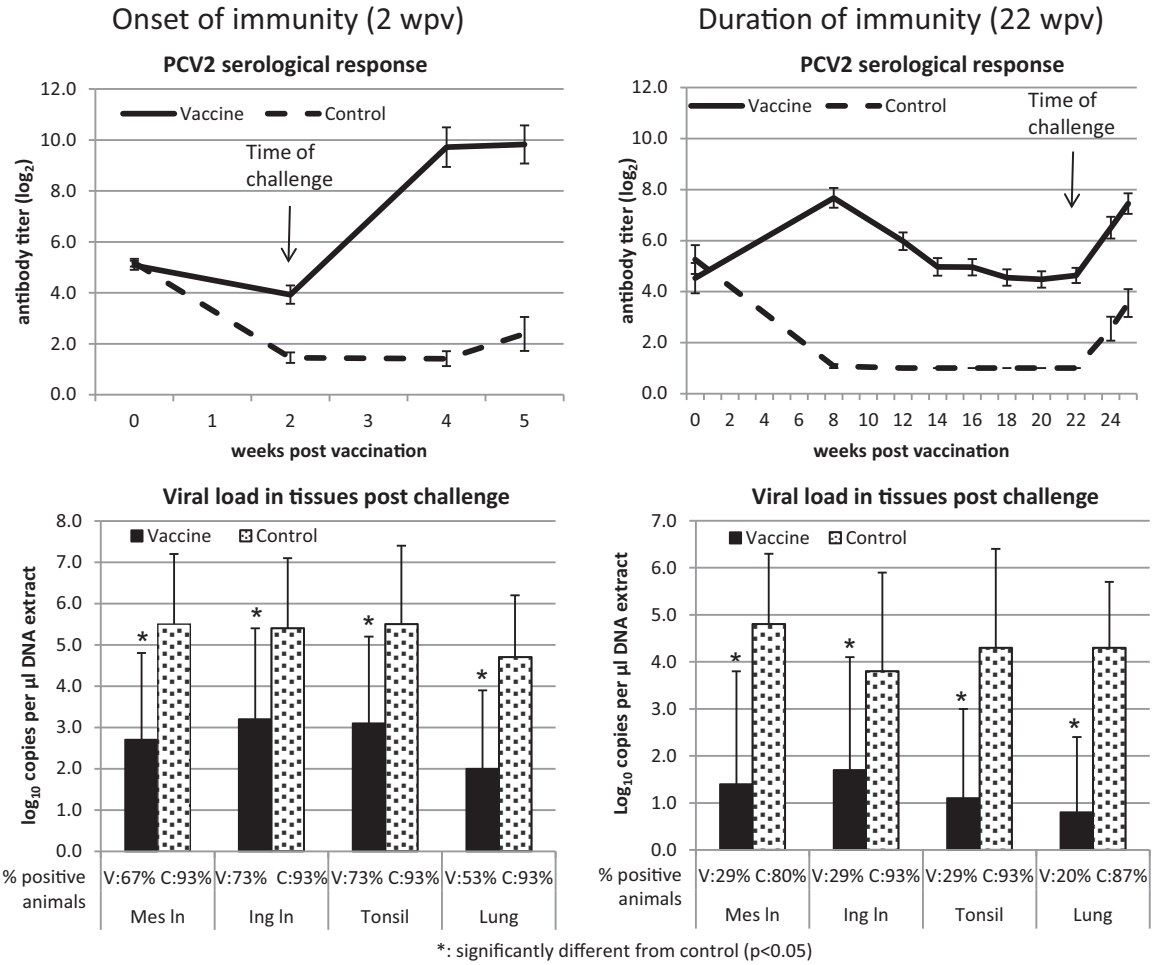

Fig. 1. Mean group anti-PCV2 antibody responses, percentage of PCV2 positive animals and mean PCV2 DNA load in tissue samples at necropsy in PCV2 challenge studies. Pigs were infected at 2 or 22 weeks post vaccination.

sow as random effect. The proportions of pigs with pleurisy (absent or present), the mortality and the morbidity were compared between the groups by the Cochran Mantel Haenszel method with production batch as classification variable. The ADWG was compared between the groups using a mixed model ANOVA. Vaccination group, production batch and gender with appropriate interactions were included as fixed effects and sow (and farm) as a random effect. The body weight at admission was included in the model as a covariate. The numbers of pigs with local or systemic reactions and the *M. hyopneumoniae* serological responses were compared with the Fisher's exact test.

3. Results

3.1. Safety studies

Results of all safety studies are summarized in Table 2.

In the laboratory safety study, none of the animals developed local or systemic reactions and no macroscopic abnormalities were observed at the injection site at necropsy. At 4 hours after vaccination, the rectal temperature of vaccinated animals was on average 1.1°C higher than in the control animals ($p < 0.001$) but returned to normal on the day after vaccination.

In the field safety study, treatment resulted in a local reaction with a maximum diameter of 1 cm in 13% of the vaccinates and 0.3 cm in 4% of the controls. These local reactions were observed at 4 hours post vaccination only and had disappeared by the next day. The numbers of piglets with a deviation from the normal general health after treatment were similar in both groups (6% and 5% for vaccinates and controls, respectively). A 1.1°C higher mean rectal temperature ($p < 0.0001$) at 4 hours post vaccination was measured in the vaccinates (40.6°C vs. 39.5°C), which returned to normal on the day after vaccination. Weight gain was not significantly different between groups during the three week observation period after treatment.

In the field safety and efficacy study, local reactions were observed in approximately 1% of the pigs in both groups. The maximum size of the local reactions in the vaccinates was 2 cm and the maximum duration was one day. A deviation from the normal general health was observed in 3% of the vaccinates and 1% of the controls. The animals mostly showed minor signs of discomfort 4 hours after vaccination.

3.2. Challenge studies

No clinical abnormalities that could be related to treatment were present in the periods between vaccination and challenge. However, some vaccinated and control pigs had lameness during the studies, most likely due to a *Streptococcus suis* infection, and in total eight vaccinates and six controls had to be euthanized for animal welfare reasons. The PCV2 challenge infection did not result in any clinical signs, but the qPCR data clearly showed infection of the various lymphoid tissues and lung (Fig. 1). Mean viral loads were in general in the order of 2-3 $\log_{10}$ lower in the vaccinated pigs, and the differences between the groups were statistically significant ($p < 0.05$). Vaccination also resulted in a significantly

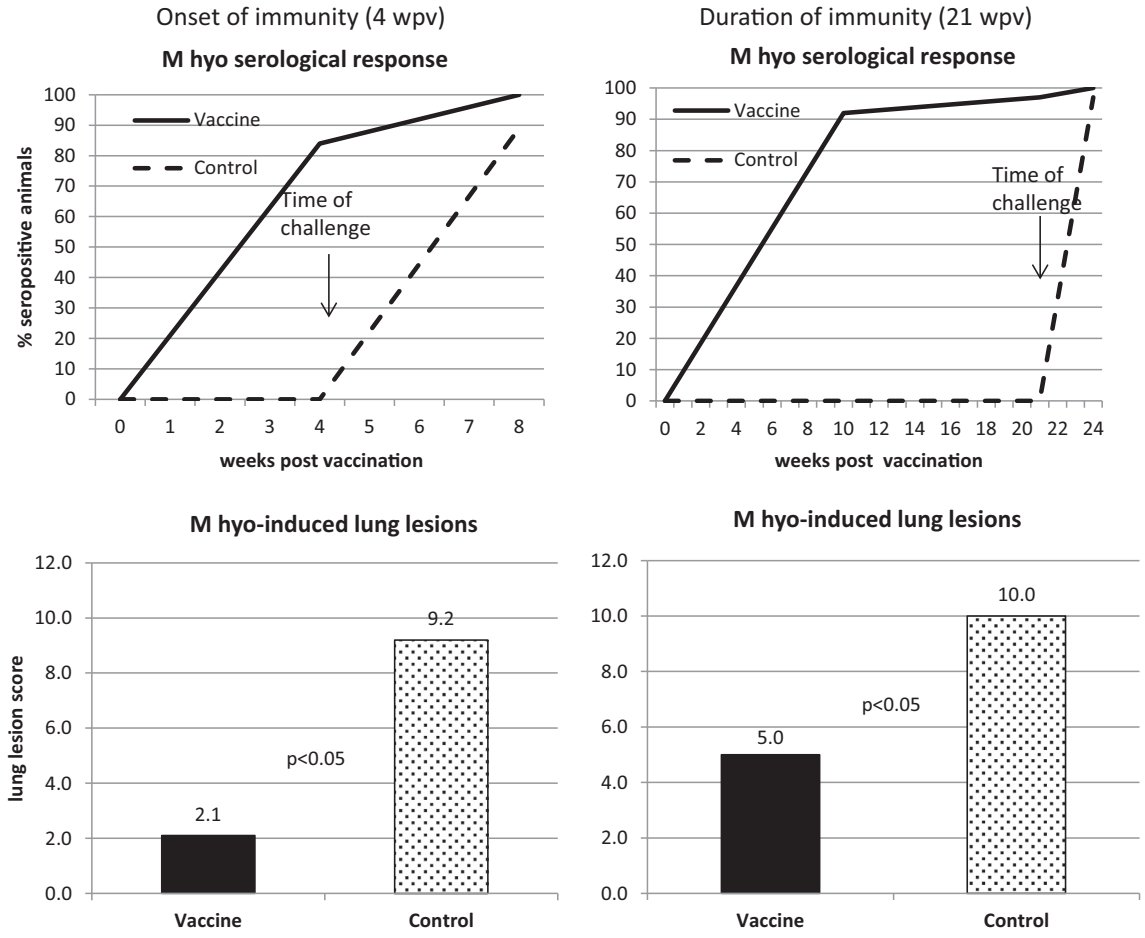

Fig. 2. Group anti-*M. hyopneumoniae* seroresponse rates and median lung lesion scores at necropsy in *M. hyopneumoniae* challenge experiments. Pigs were infected at 4 or 21 weeks post vaccination.

higher antibody level against PCV2 at 2 wpv (OOI study, $p < 0.0001$ as well as for the 0–22 wpv period (DOI study, $p < 0.0001$), compared to the control group that remained serologically negative after the decline of maternal antibody titers until the time of challenge. Following challenge, vaccinates developed an anamnestic response and the animals in the control group started to seroconvert.

A seroresponse after vaccination was also seen in the *M. hyopneumoniae* challenge experiment with 84% of animals seropositive at 4 wpv and 97% of animals seropositive at 21 wpv (Fig. 2). Almost all the control animals responded serologically to the challenge infection. At necropsy three weeks post challenge, the median *M. hyopneumoniae*-induced lung lesions in the vaccinated groups were 77% (OOI study) and 50% (DOI study) lower than in the control groups ($p < 0.05$). In both of the studies, five of the vaccinates did not have lung lesions compared to two (OOI study) and one (DOI study) of the controls. These differences were not statistically significant.

3.3. Field efficacy study

The PCV2 serological profile of the pigs in the field study (Fig. 3) is indicative for a PCV2 infection between 8 and 12 wpv. The mean antibody titers of the vaccinated animals were significantly higher than those of the controls at 4, 8 and 12 wpv ($p < 0.01$). At 8 weeks post vaccination the presence of PCV2 could be detected at low levels in control animals reaching a peak in nasal and fecal excretions at 12 wpv and in serum at 16 wpv. Compared to the control animals the viral load of the vaccinated animals (calculated as AUC) was significantly reduced by 79% ($p < 0.0001$), 70% ($p < 0.0001$) and 55% ($p = 0.0159$) in serum, nasal and fecal excretions, respectively.

As shown in Fig. 4, 46% of vaccinated animals became *M hyopneumoniae* seropositive at 4 wpv. *M. hyopneumoniae* seropositive control animals were observed at 16 wpv and coughing as a sign of *M. hyopneumoniae* infection was observed in the herd. The number of seropositive animals in the vaccinated group was significantly higher at each time point after vaccination ($p < 0.01$). At slaughter, the lung lesion scores in the vaccinated group were 46% lower than in the control animals ($p < 0.0001$). In particular, the percentage of animals with severe lung lesions (score >10) was reduced by 56%. The number of animals with pleurisy was lower in the vaccinated group (32% versus 39%), but this reduction was not statistically significant ($p = 0.121$).

Vaccination with Porcilis® PCV M Hyo induced a 34 g higher ADWG during finishing ($p < 0.0001$) and a 19 g higher ADWG during the entire study period ($p = 0.0019$) than in the control animals (Table 3). Although morbidity and mortality were both lower in the vaccinated group, the differences with the controls were not statistically significant.

4. Discussion

The present study supports that the new Porcilis® PCV M Hyo vaccine can safely be given to piglets of 3 weeks of age. The frequency of systemic reactions was very low and as these reactions

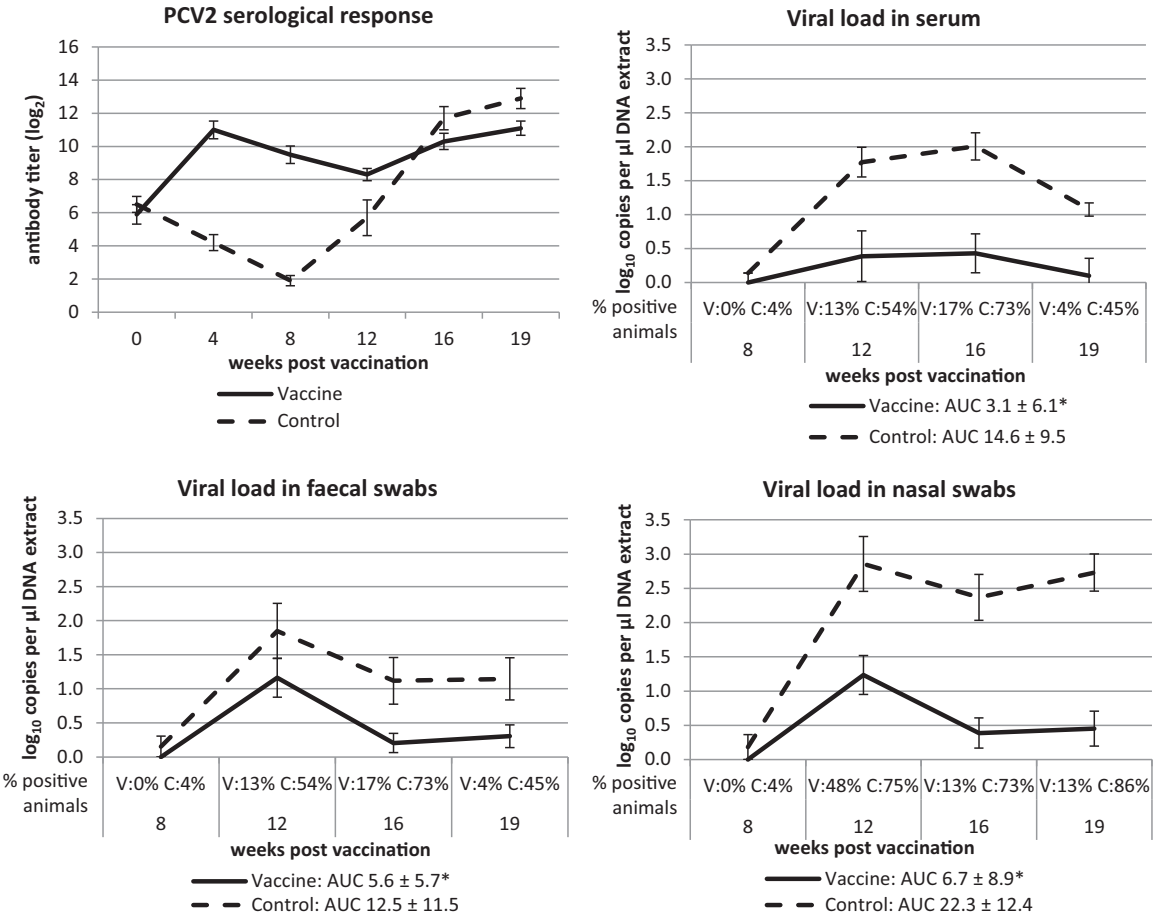

Fig. 3. Mean group anti-PCV2 antibody responses, percentage of PCV2 positive animals and mean PCV2 DNA load in serum and fecal and nasal swabs in the field efficacy study. The corresponding mean areas under the curve (AUC) are presented below the individual panels.

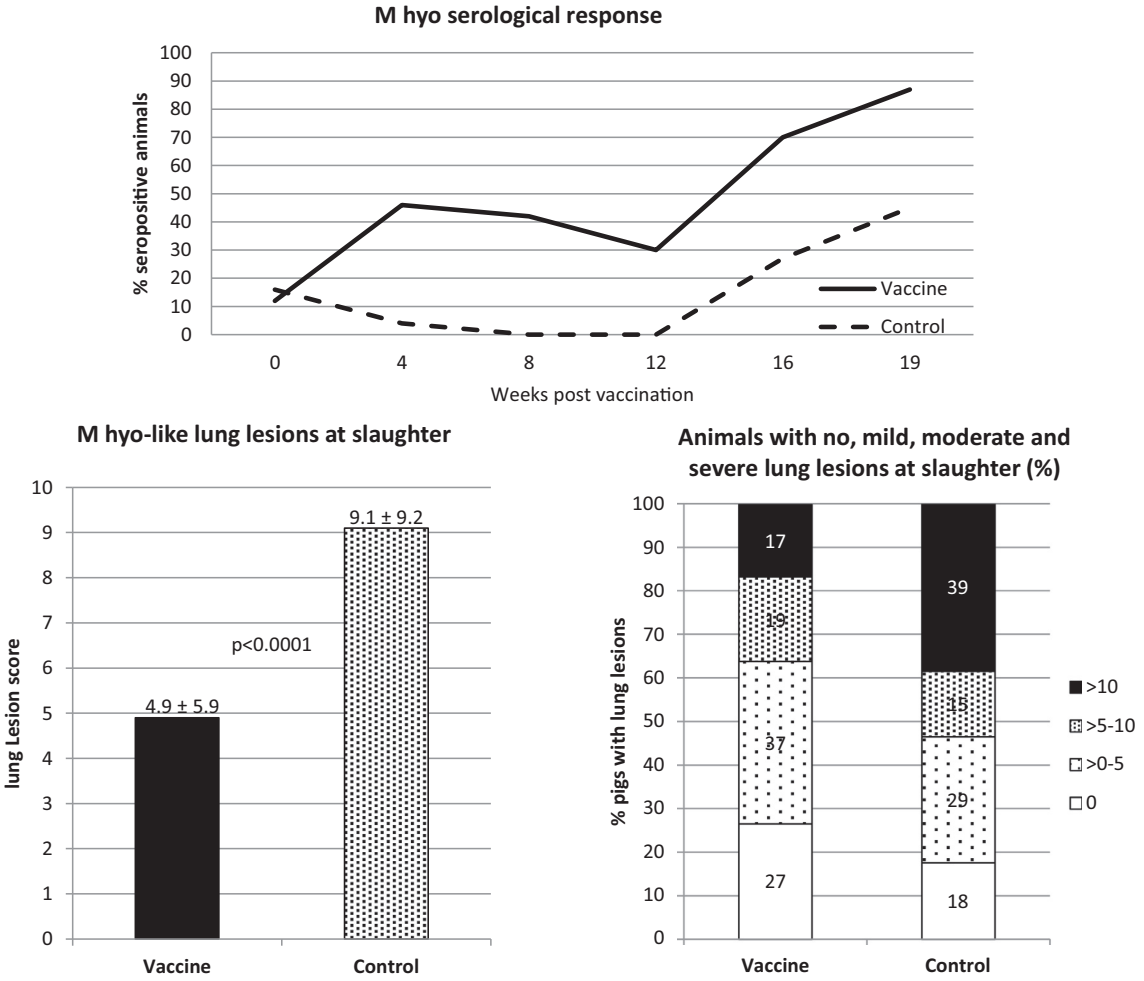

Fig. 4. Group anti-*M. hyopneumoniae* seroresponse rates, mean lung lesion scores at slaughter and lesion score distribution in the field efficacy study.

Table 3
Descriptive data of study animals and performance in the combined PCV2-M. hyopneumoniae field efficacy study

	Weeks post vaccination	Vaccine	Control	Difference[1]	p-value
Number of pigs (n)	Study inclusion	302	303		
Number of males/ females (n)		155/ 147	160/ 143		
Age of pigs (days)		17.9	17.9		
Morbidity (%)	0–19	2.6	3.3	-0.7	0.6373
Mortality (%)	0–7	1.3	1.3	0.0	0.9960
	7–19	2.7	3.7	-1.0	0.4890
	0–19	4.0	5.0	-1.4	0.5563
ADWG (g/day)	0–7	360 ± 4	369 ± 4	-9	0.0839
	7–19	757 ± 7	723 ± 7	+34	<0.0001
	0–19	612 ± 5	593 ± 5	+19	0.0019

[1] Vaccine group minus Control group

were also observed in the control groups that were injected with saline, they appear to be more treatment related than a result of vaccination. In both field studies, the vaccinates had a slightly lower ADWG than the controls in the nursery phase but the differences were not statistically significant. The local reactions were small and transient and an average increase in the rectal temperature of approximately 1°C was observed at 4 hours post vaccination. However, as the temperature returned to normal the following day and as furthermore neither the general behavior nor the feed intake (as measured by body weight at 3 or 7 weeks post vaccination in the field studies) of the animals was affected, this transient increase of rectal temperature can be considered to be an acceptable vaccine related finding. This notion is supported by the fact that an average increase of 1°C is well within the limit of 1.5°C that is allowed according to European Pharmacopoeia monograph 2448 (porcine enzootic pneumonia vaccine (inactivated)).

The experimental challenge studies indicate that the onset of immunity occurs as early as 2 weeks (PCV2) to 4 weeks (*M. hyopneumoniae*) post vaccination and lasts for at least 21 (*M. hyopneumoniae*) to 22 (PCV2) weeks. This was demonstrated by a significant reduction of the PCV2 viral load in lymphoid organs and lungs and a significant reduction of *M. hyopneumoniae* specific lung lesions. Accordingly, a single vaccination of animals at 3 weeks of age may protect fattening pigs against PCV2 and *M. hyopneumoniae* infections during the production life cycle.

The observations made during the challenge experiments were confirmed in the field efficacy trial: strong reductions in PCV2 viral load and *M. hyopneumoniae*-induced lung lesions were measured. The field efficacy study showed that vaccination with Porcilis® PCV M Hyo reduced the level of the viral load of the pigs in serum and excretions via the nasal and fecal route after infection. The PCV2 infection encountered in the field study was primarily subclinical.

The serological and virological profiling of the animals in the field efficacy study indicates that PCV2 infection started at approximately 8 wpv and an increase in the number of *M. hyopneumoniae*

seroresponders was observed between 12–16 wpv. Considering that seroconversion against *M. hyopneumoniae* generally occurs approximately 3-4 weeks post infection [15], the serological profile is indicative for an *M. hyopneumoniae* infection at around the same time as the peak of the PCV2 infection (12–16 wpv, corresponding to 15 to 19 weeks of age). In case of experimental dual infection with PCV2 and *M. hyopneumoniae*, in which PCV2 challenge was performed one week after *M. hyopneumoniae* challenge PCV2 has been shown to potentiate the severity of *M. hyopneumoniae* lesions and *M. hyopneumoniae* has been shown to potentiate the severity of PCV2 viremia [16]. The effects of a dual infection on the animal performance are therefore usually more dramatic than with any of the two pathogens alone. In the field study, this is reflected by a 34 g higher ADWG during the finishing period (time period between 10 and 22 weeks of age).

In case of dual infections with PCV2 and *M. hyopneumoniae,* it has furthermore been demonstrated that vaccination against either one of the two pathogens alone does not reduce the severity of infection of the respective other pathogen [16]. Consequently, vaccination against one of the two pathogens alone is not sufficient to protect animals from dual infections with both pathogens, highlighting the need and the benefit of combined PCV2-*M. hyopneumoniae* vaccines.

In conclusion, this is the first report to show that it is possible to develop a ready-to-use PCV2 and *M. hyopneumoniae* vaccine that can be given as a one dose product.

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