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The prevalence of PCV2 viremia in newborn piglets on four endemically infected Dutch sow farms is very low

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ABSTRACT

Porcine circovirus type 2 (PCV2) systemic disease is currently considered one of the most relevant infectious diseases in swine industry worldwide from an economical point of view. Although piglets generally become diseased between 8 and 16 weeks of age, they can be infected much earlier, even already in utero. However, data on the prevalence of PCV2 infection in newborn piglets are very variable (lower than 40 up to 82%) and most of the studies have been performed in US. In European pig farms, using group-housing systems for gestating sows, a different herd PCV2 infection and immunological status may be expected and was recently reported in Germany. If that is the current scenario in most European farms, strategies to prevent horizontal transmission become essential for the control of the infection.

The aim of our study was to determine the PCV2 prevalence in newborn piglets on 4 endemically infected farms in the Netherlands under European conditions. Eleven sows and 8 piglets per litter from 4 farms selected by their assumed PCV2 endemic infection status were sampled. Plasma from piglets was analysed with a PCV2 qPCR and serum from the sows was analysed with a commercial circovirus IgG ELSIA, circovirus IgM ELISA and PCV2 qPCR. In none of the samples from the piglets PCV2 was detected by the qPCR. None of the samples from the sows tested positive in the qPCR and circovirus IgM ELISA. The true- and apparent prevalence of IgG at herd and sow level were 0.75 and 0.81 and, 0.30 and 0.32, respectively, and no statistically significant association with sow parity was observed. These results reveal a very low prevalence of PCV2 in newborn piglets on endemically infected farms in The Netherlands, opening the opportunity of re-evaluation of the control measures applied in these farms.

1. Introduction

Porcine circovirus type 2 (PCV2) is considered to be a cause of a number of disease syndromes grouped in the so-called Porcine Circovirus Associated Diseases (PCVADs). Moreover, it is believed that PCV2 is widely spread in most of the farms worldwide (Segalés et al., 2005), thereby becoming one of the most important infectious agents in swine production nowadays from an economical point of view (Ellis, 2014). PCV2-systemic disease (PCV2-SD) is considered as the most relevant among this group (Ellis, 2014; Segalés, 2015). Diseased piglets show wasting, growth retardation and even emaciation in severe cases (Segalés, 2015). PCV2-SD often occurs in piglets of 8–16 weeks of age (Larochelle et al., 2003; Segalés et al., 2005). However, there is evidence that piglets can become infected already in utero (Andraud et al., 2009b; Dvorak et al., 2013; Rose et al., 2012; Shen et al., 2010). Nevertheless, little is known about the diaplacental transmission rate and odds of infection under field conditions (Rose et al., 2012). The prevalence of the infection in the early life of piglets is important with regard to the infection dynamics in a herd as well as for the expected efficacy of intervention measures commonly applied. A high number of early infected piglets has been associated with a higher number of infected piglets at weaning and higher odds of the disease under both field and experimental studies (Andraud et al., 2009a; Rose et al., 2009). Regarding intervention measures, low prevalence in newborn piglets might mean that cross fostering of litters attributes significantly to between litter transmission. In addition, a low prevalence at birth would provide sufficient time to apply efficacious vaccination of uninfected piglets. When facing high prevalence in newborn piglets, one would expect an opposite outcome.

Literature on the prevalence of PCV2 in newborn piglets shows very variable results. Although comparison of previously published studies is hampered by the use of different blood sampling protocols and use of different sampling matrices, US based studies showed moderate to high prevalence (up to 82% of prevalence) of PCV2 in newborn piglets

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(Dvorak et al., 2013; Fangman et al., 2014) while other American studies showed lower prevalence (below 40% of prevalence) (Gerber et al., 2012; Shen et al., 2010). Among other differences with US pig farming systems, European farms are obliged to house gestating sows in group housing systems and a more homogenous exposure of gestating sows to PCV2 may be expected. In addition, this homogeneous exposure might enhance a more homogeneous immune status of the herd, potentially resulting in a different picture in newborn piglets as the one attributed to US studies. In fact, a recently published study reported for the first time a 0% prevalence of PCV2 infection in pre-suckling piglets in German farms (Eddicks et al., 2016). These results give rise to an important debate about the current situation in European farms regarding PCV2 infection and spread in the field, on which the current control strategies should be based. If low prevalence is the current status in most of the European farms, it would also impact the study design (e.g. sample size) in future studies.

All together, the aim of this study was to determine the prevalence of PCV2 infection in newborn piglets on 4 endemically infected Dutch farms using sow group housing systems.

2. Material and methods

The experimental design and procedures were performed in accordance with European directive 2010/63EU and the Dutch Act on Animal experimentation and were approved by the Dutch Central Committee on Animal Experiments (licence AVD108002016502). Informed consent was obtained from the participating farmers.

In order to determine the expected low prevalence of PCV2 infection in newborn piglets on endemically infected farms, a cross sectional study was conducted on 4 farms in The Netherlands after selection for the specific inclusion criteria. Eleven sows per farm were blood sampled at one moment around farrowing, and 8 of their newborn piglets (8 piglets per litter) were blood sampled from the umbilical cord immediately after birth in the same day as their mothers. Samples of sows were analysed for the detection of IgM and IgG by enzyme-linked immunosorbent assay (ELISA) and samples from both, sows and piglets, were analysed by real-time quantitative polymerase chain reaction (qPCR) for the detection of PCV2 DNA.

2.1. Sample size calculation

For this study a sampling frame on three levels was determined. First the number of litters and the number of piglets within litters within farm needed to be determined. Next the number of farms on which the sampling was repeated had to be determined. The number of farms to sample was determined based on expert knowledge, average number of farms sampled in literature and considering financial and logistic constraints. Whereas strict inclusion criteria for the farms should prevent classification bias, four farms were selected to account for the between farm variation.

Secondly, the number of litters and the number of piglets within litters to sample were determined using simulation with R 3.4 (The R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2013) and additional library VGAM (See Supplementary material (S1) for script). For the simulations it was assumed that a considerable lower prevalence than previously reported outside the EU, p = 0.3 comparable with Gerber et al. (2012), should be accurately estimated (Dvorak et al., 2013; Fangman et al., 2014). As prior knowledge on the variation of infection status for PCV2 for piglets between litters was unavailable, it was assumed that the infection probability for individual piglets followed a beta-binomial distribution with p = 0.3 and a variation of infection between litters with rho = 0.5, similar to values found for *Actinobacillus pleuropneumoniae* infection at weaning (Tobias et al., 2014). For the simulations of the power with a given sampling frame it was assumed that the upper confidence limit of the prevalence estimate should be below p = 0.4 in 0.8 of the simulations. For each combination of a number of litters and number of piglets per litter to be sampled a simulation was run. The simulations were first repeated 1000 times to obtain a frequency distribution of prevalence estimates with a 95 percentile interval. Subsequently, the whole procedure was repeated 500 times to obtain a frequency distribution for the 0.975 percentile of the prevalence estimate procedure. Eventually the combination with the lowest total number of piglets was chosen with which an accurate (0.3 (95%CI: 0.2–0.4) estimate of PCV2 prevalence could be determined. The number of litters to sample appeared not to be very sensitive to uncertainty in the assumption of rho as long as at least 6 pigs per litter were sampled and at least 8 litters were sampled. Sample size calculations returned that when sampling from 11 litters and 8 piglets within each litter the minimum number of piglets per farm to sample was 88.

2.2. Farm and animal selection

Four commercial Dutch breeding farms were selected according to the following criteria: *i*, history of infection of PCV2 but no current clinical complaints; *ii*, multiplier herds with more than 150 sows group-housed during gestation (more than 50 sows per group); *iii*, self-raising of replacement gilts; *iv*, no standard sow vaccination against PCV2; *v*, 1-week batch system. The 4 farms were not related in terms of genetics or trade, and the farms were located 5–30 kilometres from each other. All farms routinely performed PCV2 vaccination of piglets at 3 weeks of age but no sow vaccination was performed. Information about the farms included in the study is shown in Table 1.

11 litters per farm were selected and stratified by parity in four groups: gilts, second parity, third and fourth parity and fifth or higher parity (Table 1). Within these groups, litters were selected based on convenience for farrowing when researchers were present. Within litters, 8 live-born piglets were selected for sampling in order of birth.

2.3. Sample collection

2.3.1. Piglet sample collection

A protocol for sampling was set up in order to obtain blood samples from the umbilical cord of newborn piglets during, or immediately after, expulsion before suckling. In order to minimise PCV2 environmental contamination, a clean paper cloth was placed on the floor behind the sow and gloves were changed for restraining and sampling of each piglet. In order to reduce the risk of bacterial infection of the umbilical cord, ethyl alcohol (70%) was sprayed on the umbilical cord before sampling. The piglet was restrained and presented to the sampler. Blood was collected from an umbilical vessel using an EDTA S-monovette® 1.2 mL k3E and safety needles 22G × 1½ in. (both Sarstedt B.V., Nümbrecht, Germany). Once the blood was collected, the umbilical cord was sprayed with Iodine solution (Betadine®, Meda Pharma, Amstelveen, The Netherlands) and checked for bleeding. The piglet was then dried with the paper cloth and placed gently near the mammary glands of the sow. Samples were labeled and stored at 4 °C until processing. A total of 352 blood samples were collected from live newborn piglets.

Table 1
Relevant characteristics of sampled farms and animals included in the study.

Farm	# Sows	# Farrowing sows/week	# Sampled sows per parity				# Sampled piglets
			1st	2nd	3rd–4th	> 5th	
1	190	10	2	3	3	3	88
2	450	20	2	2	4	3	88
3	675	27–37	2	2	3	4	88
4	350	16	3	3	3	2	88

2.3.2. Sow sample collection

Once the farrowing ended and after letting all the piglets suckle, blood samples were taken from the sows using a 9 mL serum-gel monovette (Sarstedt B.V., Nümbrecht, Germany).

2.4. Diagnostic test methods

Blood samples from the piglets were centrifuged (1500g × 10 min) and the obtained plasma was analysed at GD Animal Health Service (Deventer, the Netherlands). After a preliminary validation, samples were tested by an in house real-time qPCR targeting the PCV2 capsid protein gene. The PCR based on the Fluorescence Resonance Energy Transfer (FRET) principle contained per sample 10 pmol of PCV2 forward primer 1422 (GGG CCA GAA TTC AAC CTT AAC CT), 10 pmol of the PCV2 reverse primer/probe iLC (CTC TCC CGC ACC TTC GGA (LC red 640) TAT), and 6 pmol of the PCV2 probe FLw (5CGT TYT GAC TGT GGT WSS CTT GAY AGT – FL fluorescein), 2 µL Light-Cycler-FastStart DNA Master Hybridization mix (Roche Applied Science, Penzberg, Germany), 4 mM magnesium chloride, 0.5 µM of each primer and 0.15 µM of each probe. Cycling parameters were 95 °C for 10 min and 45 cycles at 95 °C for 10 s, 58 °C for 12 s, and 72 °C for 20 s. For standard curve, serial dilutions of plasmid (PCV2 cloned in pCR21) of 1–10^{11}/µl copies were used. Linear dynamic range at which reliable quantification was possible was obtained by dilution experiments and spiking with known amounts of PCV2 virus. It ranged from 2.0 × 10^4 to 2.0 × 10^{10} PCV2 viral DNA copies/ml. The limit of detection for the PCV2 PCR for serum was < 100 PCV2 viral DNA copies/ml. Sensitivity and specificity of the qPCR on plasma was assumed equal to the characteristics of the test when performed on serum (0.95 and 0.965, respectively) (Wellenberg et al., 2004). The threshold Ct value for the PCR is 35 and results above the threshold value were considered as negative. Sow serum was tested by the same qPCR as well as for IgG and IgM antibodies using the INgezim Circovirus IgM/IgG ELISA (INGENASA, Madrid, Spain) according to the manufacturer's instructions. Samples with S/P values above or equal to 1 were considered positive (IgG or IgM antibodies demonstrated). Point estimates of the sensitivity and specificity of the ELISA are 0.93 and 1 respectively (Pileri et al., 2014).

2.5. Statistical analysis

Apparent prevalence, true prevalence and confidence intervals of PCV2 infection tested by qPCR and ELISA in piglets and sows were estimated using Epitools from Ausvet® (Sergeant, 2017). Wilson's and Blaker's confidence intervals were used for the apparent and true prevalence estimations, respectively (Brown et al., 2001; Lang and Reiczigel, 2014). A Pearson's Chi-square test was applied using R software 3.4 (The R Foundation for Satistical Programming, Vienna, Austria) in order to study the association between the parity and the prevalence of IgG in sows. The level of significance (p-value) was set at 0.05.

Given negative results, the maximum possible prevalence (MPP) of infection in the sow population was estimated in the total population considering the sensitivity of the test as follows:

$$MPP = \frac{1 - (1 - CL)^{\frac{1}{n}} \times \left(N - \frac{n-1}{2}\right)}{Se \times N}$$

where CL is the level of confidence, n is the sample size, N is the population size and Se is the sensitivity of the test (Cannon and Roe, 1982)

3. Results

The herd and animal apparent- and true prevalence estimated based on the qPCR and circovirus IgM/IgG ELISA are shown in Table 2.

None of the 352 analysed samples from the piglets resulted positively by the qPCR. None of the 44 analysed samples from the sows tested positive in the qPCR. Given all negative results, the MPP of infection in the sow population was estimated at 0.07.

None of the 44 sera analysed showed positive results in the circovirus IgM ELISA. However, 13 sera out of the 44 tested positive in the circovirus IgG ELISA; none at farm 1, three at farm 2, six at farm 3 and four at farm 4. The apparent- and true prevalence considering only the three positive farms were 0.33 (0.20–0.50) and 0.36 (0.21–0.54), respectively. When testing the association between the percentage of circovirus IgG ELISA positive sows and the sow parity, no significant association was found when including neither data from the three positive farms together nor individually per farm. Due to the negative IgG ELISA results in Farm 1, a subsequent larger random sampling of 67 sows was analysed and an apparent prevalence of 0.19 was found.

4. Discussion

The objective of this study was to determine the prevalence of PCV2 viremia in newborn piglets on 4 PCV2 endemically infected Dutch farms. The results show that viremia in PCV2 newborn piglets is very rare on farms meeting the inclusion criteria of this study. These results are in accordance with another recently published study that showed that no PCV2 DNA was detected in any of the pre-suckling piglets and that the prevalence in sows was ~1% in southern Germany pig farms (Eddicks et al., 2016). However, our study is of additional value because farms were selected by their assumed PCV2 endemic status based on several criteria (see "Material and Methods"), while Eddicks et al. (2016) sampled randomly selected farms, regardless the PCV2 status (Eddicks et al., 2016). In the latest case, it is then difficult to relate the PCV2 prevalence in newborn piglets and the PCV2 infection status of the farm (absence of infection, endemic infection or epidemic infection). However, considering the results of our study, one could challenge the assumption of the study farms being endemically infected. "Endemic occurrence" of a given disease describes "the usual frequency of occurrence of a certain disease or the constant presence of the disease. It can also be applied to the presence of infectious agents and to levels of circulating antibodies" (Thrusfield, 1995). Nowadays PCV2 infection is most commonly present as a subclinical form (Nielsen et al., 2017; Segalés, 2012). That is why in our study, farms with history of infection of PCV2 but no current clinical complaints were selected. In other studies, despite the lack of clinical presentation of PCV2-SD, PCV2 DNA was detected, although it is usually associated with lower viral loads, supporting our assumption of no direct relation between PCV2-SD manifestation and PCV2 detection (Feng et al., 2014; Nielsen et al., 2017; Shen et al., 2010).

In order to avoid recent introduction of the virus and thus an epidemic outbreak, only farms using a self-raising of replacement gilts were selected. In such systems and matching the definition of "endemic disease", one would expect antibodies in the replacement gilts in an endemic situation, meaning that the virus is constantly circulating within the herd. In the three positive farms, the average prevalence in sows was 0.33 using the circovirus IgG ELISA test. Surprisingly, all sows sampled from one farm (Farm 1) resulted negative to this ELISA. However, in a subsequent larger random sow sampling the presence of IgG antibodies was demonstrated with the same ELISA test, resulting in an apparent prevalence of 0.19. In the three circovirus IgG ELISA initially positively tested farms, no statistical association between antibody detection and the sow parity was observed, and some gilts tested positive to the ELISA as well, supporting the assumption of an endemic status.

Assuming the hypothesis of endemic infection, the negative qPCR results in piglets could be explained by a viremia below detectable levels or by the lack of viremia at the moment of sampling. It has been evidenced that vaccination programs may reduce the PCV2 viremia even to undetectable levels (Dvorak et al., 2016; Feng et al., 2014; Fort et al., 2009; Nielsen et al., 2017; Segalés, 2015; Seo et al., 2014). The study of Dvorak et al. (2016) showed a markedly decreased antibody

Table 2
Herd, sows and piglets apparent- and true prevalence estimated using Circovirus IgG ELISA, Cirovirus IgM ELISA and qPCR PCV2.

Test	Herd (n = 4)		Sows (n = 44)		Piglets (n = 352)	
	AP	TP	AP	TP	AP	TP
Cirovirus IgG ELISA	0.75 (0.30–0.95)	0.81 (0.32–1)	0.30 (0.18–0.44)	0.32 (0.19–0.48)	NA	NA
Cirovirus IgM ELISA	NA	NA	0 (0–0.08)	0 (0–0.09)	NA	NA
qPCR PCV2	NA	NA	0 (0–0.08)	0 (0–0.05)	0 (0–0.01)	0 (0–0)

AP, apparent prevalence; TP, true prevalence; n, sample size; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; NA, not applicable.

prevalence and PCV2 DNA prevalence in sows and finishing pigs after 6 years of vaccination strategy implementation (Dvorak et al., 2016). In addition to that, in the study of Feng et al. (2014) ELISA OD values in piglet's serum from seropositive sows decreased during a period of extensive vaccination (Feng et al., 2014). In order to prevent interference and confounding in both antibody titers and PCV2 viremia by vaccination, only farms not applying standard sow vaccination against PCV2 were selected for our study. However, unfortunately no farms could be found that did not perform PCV2 vaccination in the piglets, and the effect of this measure on farm level in our study remains unknown. Finally, the absence of PCV2 DNA detection in serum does not exclude the possibility of latent PCV2 infection. In fact, some studies have indicated that the thymus of pig fetuses may harbor latent PCV2, and that at a certain point in time reactivation may occur resulting in productive infection (Klausmann et al., 2015; Sydler et al., 2016). This theory seems unlikely to explain the total absence of detection of PCV2 in serum in our study, since studies published up to now detected PCV2 in newborn piglets pre-suckling using blood samples (Dvorak et al., 2013; Fangman et al., 2014; Feng et al., 2014; Gerber et al., 2012; Shen et al., 2010). However, differences in the PCV2 infection status and management could again play a role here. Nevertheless, these findings may suggest that in similar circumstances as in the farms under study, serum may not be the target matrix to study the PCV2 infection in newborn piglets, and some other samples such as thymus would be preferred. Whether such sampling, including sacrificing piglets, is ethically or economically acceptable for the farmer weighs out to the research outcome is to be discussed.

Even assuming that the study farms were endemically infected at some time, epidemiological studies showed that factors such as passive immunity through colostrum or homogeneous PCV2 vaccination in piglets decreased the reproduction ratio to values around 1 (Rose et al., 2016, 2007), leading to the hypothesis of a breakdown of the endemic equilibrium and a spontaneous fade-out of the infection in those farms.

Given the high frequency of negative results, the sample size becomes a limitation to detect and accurately estimate an even lower prevalence in newborn piglets, as it was the case in Farm 1 with the IgG prevalence estimation. Given all negative results in the qPCR, the MPP of infection in the sow population was calculated as 0.07. To estimate the MPP in newborn piglets the litter effect should be taken into account. However, to our knowledge the methodology to calculate the MPP accounting for a random group (e.g. litter) effect is unavailable.

Finally, the external validity of our results is limited to farms matching the inclusion criteria of our study, which support the likelihood of a low level of PCV2 virus circulation. Therefore our results cannot easily be extrapolated to farms that have an increased risk of introduction of PCV2, risk of active shedding of PCV2 or those that hinder the exposure of gestating sows and thereby prevent boostering of immune system. However, the relevance of the observed low frequency of PCV2 viremia in newborn piglets, supports the importance of preventing horizontal transmission after birth and seems to provide ample time to implement effective vaccination in piglets from positive farms. In conclusion, the results of our study, reveal a lower than expected prevalence of PCV2 in newborn piglets on endemically infected farms in the Netherlands meeting the inclusion criteria, opening the opportunity of re-evaluating control measures applied in these farms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prevetmed.2018.03.001.

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