Reproductive failure in an Austrian piglet-producing farm due to porcine circovirus genotype 2d

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CASE REPORT

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ABSTRACT

Infections of pigs with porcine circovirus type 2 (PCV2) can lead to various clinical conditions including reproductive disorders (PCV2-RD). In general, a transplacental infection of fetuses leads to mummification and stillbirth. So far, PCV2-RD has mainly been described in specific-pathogen-free (SPF) herds or farms with a high proportion of gilts. From December 2018 to February 2019, a high abundance of mummified fetuses (15.5%) was observed in two farrowing groups in an Austrian piglet-producing farm. PCV2 DNA was detected using qPCR in organs of all six investigated fetuses (2.07×10^8–1.09×10^{12} PCV2) genome equivalents/g tissue and via *in situ* hybridisation in organs from five fetuses, while histologic lesions were not observed in a single fetal heart. All isolates were sequenced and identified as PCV2d. After the implementation of a regular vaccination of all sows against PCV2, the abundance of mummified fetuses dropped to 3.5% in May 2019. In contrast to previous reports about PCV2-RD, this farm was neither an SPF herd nor a start-up herd with a high proportion of gilts. The implementation of regular PCV2 vaccination helped to reduce the abundance of mummified fetuses substantially.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is associated with several clinical conditions including reproductive failure, like stillbirths and mummified piglets (West et al., 1999; Brunborg et al., 2007; Madson et al., 2009). Over the last few years the number of published cases in Europe has been rather low (Brunborg et al., 2007; Hansen et al., 2010; Oropeza-Moe et al., 2017). Most reports about PCV2-associated reproductive disorders refer to farms with a seronegative PCV2 status or start-up herds with a high proportion of gilts (Brunborg et al., 2007; Hansen et al., 2010; Oropeza-Moe et al., 2017). This is most likely due to the high prevalence of PCV2 in sows and probably also due to the frequently applied PCV2 vaccinations of

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piglets (Opriessnig et al., 2007; Karuppannan and Opriessnig, 2017). Since the replication of PCV2 depends on the S phase, cells with a high mitotic rate are the main target of the virus (West et al., 1999). Due to its high tropism for fetal cardiomyocytes, PCV2 is most likely to be detected in heart tissue (Pensaert et al., 2004). The most consistent microscopic changes include myocardial degeneration, fibrosis and non-suppurative to necrotising or fibrinous myocarditis (West et al., 1999; O'Connor et al., 2001; Sanchez et al., 2001). Diagnostic approaches include the identification of PCV2 DNA in the hearts via PCR as well as *in situ* hybridisation (ISH) or immunohistochemical methods for the visualisation of PCV2 DNA/antigen in the lesions. According to Segalés (2012), the diagnosis of PCV2 reproductive disease (PCV2-RD) can only be done properly if three criteria are fulfilled, including (1) clinical signs with high numbers of mummified fetuses, stillborn or weak-born piglets, (2) microscopic lesions in the fetal myocardium, and the (3) presence of high amounts of PCV2 in myocardial lesions and other fetal tissues (Segalés, 2012). Recently, it has been doubted if the presence of microscopic lesions in individual fetuses is a necessary criterion for the diagnosis of PCV2-RD (Unterweger et al., 2021).

CASE PRESENTATION

The case herd is a commercial piglet producing farm with 105 sows (Large White × German Landrace crossbred) located in Upper Austria and is operating in a three-week batch production system. Approximately three weeks after farrowing, sows are vaccinated against porcine parvovirus 1 (PPV1) and *Erysipelothrix rhusiopathiae* (Parvoruvac®, Ceva Santé Animale, France). Moreover, all sows are vaccinated against the porcine reproductive and respiratory syndrome virus 1 (PRRSV1) every five months using a live attenuated PRRSV1 vaccine (Porcilis® PRRS, MSD Animal Health, Netherlands). Gilts are purchased every nine weeks from a single PRRS-free farm, where they are vaccinated against PCV2 (Suvaxyn® Circo, Zoetis Belgium SA, Belgium) on their 28th day of life. All gilts are kept in quarantine units for six weeks, where they are immunised against PPV1, *E. rhusiopathiae* and PRRSV1, and synchronised with Altresyn® (Ceva Santé Animale, France). The stable was built in 2005 and consists of four gestation units within the same room with fully slatted floors, a separate insemination area, two farrowing units, three nursery units and one quarantine. All pens in the nursery and farrowing units are washed and disinfected with formaldehyde and glutaraldehyde between every batch, while all-in/all-out is not possible in the gestation units, which are only washed but not disinfected. Semen is acquired from an official boar station which is regularly tested for PRRSV. One teaser boar (Piétrain) which was purchased in early 2018 is kept in an additional pen in the service centre. Sows are fed with commercial gestation and lactation feed using automatic feeding in the gestation units. All piglets receive their first vaccination against *Mycoplasma*

hyopneumoniae (Suvaxyn® M. hyo, Zoetis, Spain) on their 3rd day of life and their second vaccination on their 21st day of life together with a PCV2 vaccine (Suvaxyn® Circo, Zoetis Belgium SA, Belgium).

In December 2018, fertility problems started with mummifications of two entire litters within the same farrowing group consisting of 15 sows (sows 007 and 228) (Table 1). While reproductive disorders were not recorded in the consecutive farrowing group in January, they reappeared in the following group in early February 2019 (sows 215 and 235), when mummification was observed again in two out of 15 litters. In contrast to the incidence in December, this time a high variation of the crown–rump lengths amongst the fetuses was observed but not documented. Photos from two litters with a high abundance of mummified fetuses have been taken from the two consecutive farrowing groups in late February (sow 060) and March (sow 018) (Figs 1a & 1b). Altogether, an increase of mummified fetuses was observed in six out of sixty sows (10%) over a period of twelve weeks (Table 1).

During the period from December 2018 to March 2019 neither an increase of the return to oestrus rate nor prolonged farrowing were observed. The number of sows, which had to be treated due to an elevated inner body temperature or other clinical signs of postpartum dysgalactia syndrome, was not increased compared to previous farrowing groups. The average total litter size including liveborn, stillborn and mummified fetuses accounted for 13.9 piglets in December and 14.0 piglets in February and was not decreased compared to previous farrowing groups in October (14.6) and November (13.6). Prior to the emergence of reproductive problems, the abundance of mummified fetuses per month accounted for 0.5% in October and November 2018. However, in December and February the percentage of mummified fetuses was 15.5% and 15.4%, respectively (Table 2). Three of the six affected sows (sows 007, 060 and 018) were inseminated successfully and neither mummified fetuses nor rebreeding occurred again, while the other three sows were replaced immediately after gestation due to their age. Clinical signs were not observed during the suckling period of liveborn piglets from any litter of the affected farrowing groups.

In March 2019, six mummified and stillborn fetuses from one litter (sow 018) were sent to the University Clinic for Swine, Vetmeduni Vienna, for a diagnostic workup.

Table 1. Number of mummified and liveborn fetuses of six sows with reproductive disorders

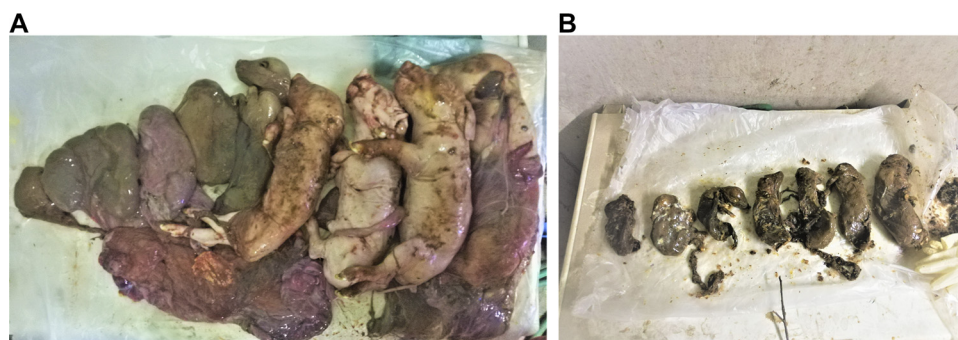| ID sow	Date of parturition	Parity	Number of mummified fetuses	Number of liveborn piglets
228	22 December 2018	8th	12	0
007	22 December 2018	5th	19	0
235	2 February 2019	8th	10	2
215	2 February 2019	9th	10	3
060	22 February 2019	2nd	15	0
018	13 March 2019	5th	7	6

Fig. 1A and 1B. Litters of two sows (060 and 018)

Table 2. Reproductive performance from July 2018 to June 2020

Farrowing period	Average rate of mummified fetuses	Average number of liveborn piglets/litter
Q3/2018	0.7%	13.4
Q4/2018	4.9%	12.1
Q1/2019	8.8%	12.2
Q2/2019	2.8%	12.8
Q3/2019	1.4%	12.7
Q4/2019	1.6%	12.9
Q1/2020	1.9%	12.9
Q2/2020	1.4%	12.9

Q1: January – March, Q2: April – June, Q3: July – September, Q4: October – December.

Prior to sampling the organs of fetuses, the crown–rump length was measured from each fetus (Table 3). According to the crown–rump length, all but fetus 5 had died in the last trimester of gestation. Routine diagnostic PCRs were negative for PRRSV1, PPV1, PCV3, encephalomyocarditis virus (EMCV), porcine teschovirus (PTV), *Leptospira interrogans* and *Chlamydia* spp. (Table 4), while PCV2 DNA was detected by qPCR in all heart samples and one lung tissue sample, which was taken by mistake from one fetus instead of the heart (Table 3). One PCR product (Fetus 3) was further sequenced. The sequence was deposited to the GenBank and assigned to accession number OM460170. In phylogeny reconstruction, the putative new virus (AUT174/2019) clustered with other PCV2d sequences (Fig. 2). PCR and phylogenetic analysis were performed as described before (Eddicks et al., 2015).

After obtaining the PCR results, heart, lung and kidney samples from all six fetuses which were stored in formalin were investigated by ISH for PCV2-DNA as previously described (Unterweger et al., 2021). ISH revealed multiple virus signals (+++) in three out of six heart samples (Fig. 3b), moderate virus signals in fetus 4 and few virus signals in fetus 6 (+). One heart sample was ISH negative

Table 3. Quantification of porcine circovirus DNA in infected organs

Fetus ID	Crown–rump (cm)	Tested organ	PCR PCV2 GE g^{-1} tissue	ISH[*]
Fetus 1	24	Heart	3.38×10^{11}	Highly positive (+++)
Fetus 2	23	Heart	2.07×10^{8}	Negative
Fetus 3	22	Heart	1.09×10^{12}	Highly positive (+++)
Fetus 4	18.5	Lung	3.71×10^{11}	Highly positive (+++)
Fetus 5	16	Heart	1.43×10^{11}	Highly positive (+++)
Fetus 6	27	Heart	9.60×10^{9}	Positive (+)

[*]*in situ* hybridisation.

Table 4. Molecular investigations of fetal organs

Pathogen	Tested organ	Method	Result	PCR assay according to
PRRSV	Thymus	RT-PCR	Negative	Fetzer et al. (2006)
PPV	Lung, Liver, Placenta	PCR	Negative	Opriessnig et al. (2011)
PCV2	Heart/Lung	qPCR	Positive	Opriessnig et al. (2003)
PCV3	Heart	qPCR	Negative	Palinski et al. (2017)
EMCV	Heart	RT-PCR	Negative	Pérez and de Arce (2009)
PTV	Lung, Liver, Placenta	RT-PCR	Negative	Palmquist et al. (2002)
Leptospira interrogans	Lung, Liver, Kidney	PCR	Negative	Letocart et al. (1997)
Chlamydia spp.	Lung, Liver, Kidney	PCR	Negative	Kauffold et al. (2006)

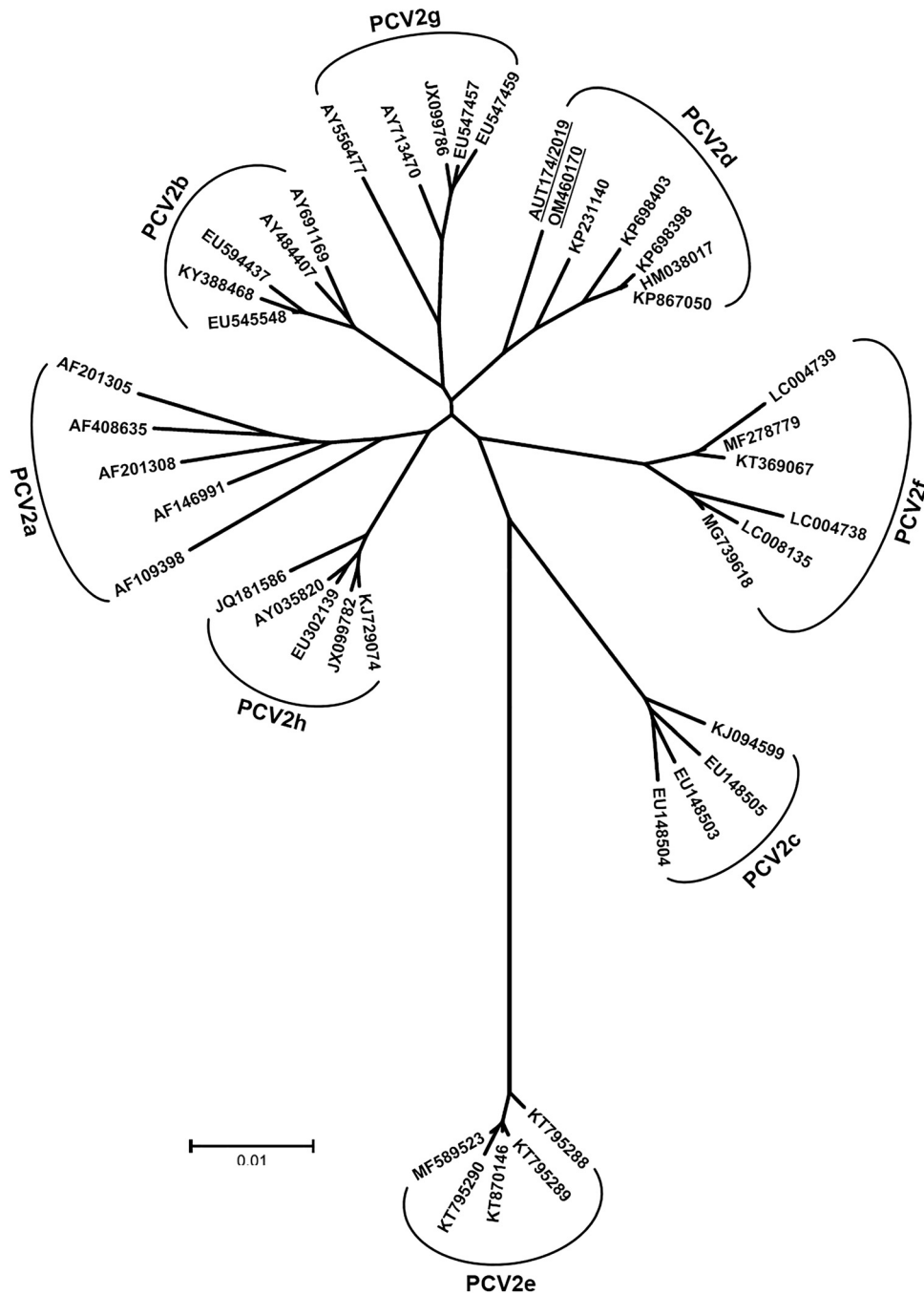EMCV = encephalomyocarditis virus; PTV = porcine teschovirus.

Fig. 2. Phylogenetic analysis based on the complete genome of selected porcine circovirus 2 reference strains. The porcine circovirus 2 sequence detected in this study is underlined. The tree was constructed using the neighbour-joining method (P-distance model; 1,000 bootstraps). The scale bar indicates nucleotide substitutions per site

(fetus 2; 2.07×10^8 PCV2 GE g^{-1}). Additionally, lung tissue from fetus 4, which was highly positive by qPCR, also revealed multiple virus signals via ISH (+++). Positive virus signals (++) were also detected in the lungs of three other fetuses (fetus 1, 3 and 5) and the kidney of one fetus (+). Since neither lung nor heart tissue of fetus 2 and 6 showed strong virus signals, thymus samples of those two fetuses were investigated as well. The thymus of fetus 2 showed a few PCV2-positive signals (+), while the thymus of fetus 6 was PCV2 negative. Unfortunately, fetal organ samples

showed a significant decomposition. No evidence of an inflammatory process was found in any examined organs in the histopathological examination (Fig. 3a).

Based on the clinical signs, postmortem examinations and the highly positive qPCR results, the herd-attending veterinarian decided to vaccinate all gilts and sows against PCV2 (Suvaxyn® Circo, Zoetis Belgium SA, Belgium) in March 2019. This vaccination was carried out once as a mass vaccination. Afterwards, gilts were routinely vaccinated once in the quarantine unit and sows were revaccinated three
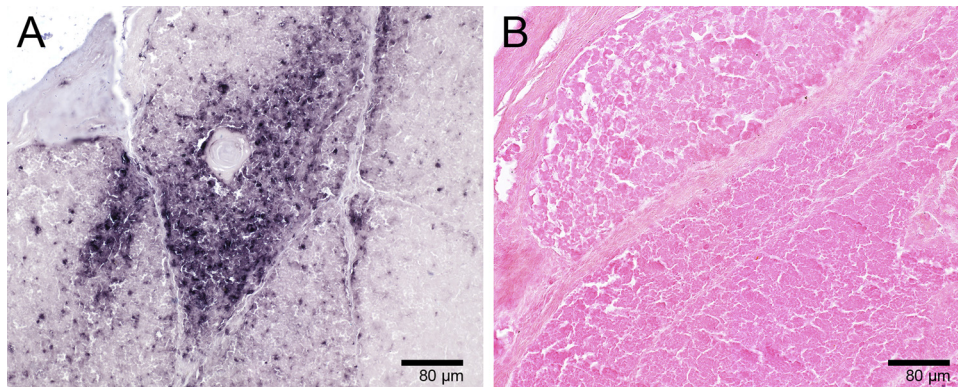
Fig. 3A. In situ hybridisation of fetal heart tissue (Fetus 3) with several porcine circovirus 2 positive signals in myocardial tissue.
3B. Histologic section (haematoxylin and eosin staining; bar 80 µm) of the same area (F 3) without lesions.

weeks after farrowing. Until May 2019, the abundance of mummified fetuses dropped to approximately 3.5% and further to 1.5% in June. PCV2 DNA could not be detected in altogether 18 fetal hearts, which were investigated between June 2019 and February 2020.

DISCUSSION

Although PCV2-RD was first described more than two decades ago (West et al., 1999), the exact pathogenesis of this manifestation of PCV2 infection and its specifics of transmission are still poorly understood (Park et al., 2005; Madson and Opriessnig, 2011). In general, viraemia in sows can result in vertical transmission to fetuses. However, it is unclear to what extent vertical PCV2 transmission occurs under field conditions, especially if sows are infected subclinically (Madson and Opriessnig, 2011). Interestingly, mummification was only observed in litters from sows in the second parity and beyond, but not in litters from gilts. This stands in contrast to previous reports of PCV2-RD, claiming that PCV2-RD was mainly observed in gilts (West et al., 1999; Hansen et al., 2010). Furthermore, the number of purchased gilts was not increased prior to the incidence.

While there are several case reports on PCV2-RD referring to the detection of PCV2a and PCV2b (Shen et al., 2010), this example is one of the first case reports with reproductive disorders caused by PCV2d. However, since there might be no differences in the outcome of other PCV2-associated diseases depending on the genotype, reproductive disorders caused by PCV2d might not be distinguishable from those caused by PCV2a or PCV2b. In general, these findings are not surprising, since PCV2d has replaced PCV2b as the most common PCV2 genotype in Austrian swine stocks (Weissenbacher-Lang et al., 2020). However, this could provide a potential explanation, why gilts did not express reproductive disorders, since they might have already been in contact with PCV2d in the nursery, whereas sows with more parities might have had contact only with PCV2b. Nevertheless, this remains a speculation, since sequencing was not performed from older samples from the

farm. Since there was no high similarity to any previously described sequence, the possible source could not be determined.

Besides subclinical infection, PCV2 can be also transmitted via the semen of infected boars (Larochelle et al., 2000; Kim et al., 2004; McIntosh et al., 2006; Madson et al., 2009). Boars are able to shed PCV2 continuously for extended time periods without expressing clinical signs or changes in the quality of semen parameters (McIntosh et al., 2006; Madson et al., 2009). Moreover, artificial insemination of sows with PCV2-spiked semen resulted in fetal infection, which was clearly demonstrated by the presence of PCV2 antigen in the myocardium of stillborn and mummified fetuses as well as liveborn piglets (Madson et al., 2009). Besides semen, transmission could have also occurred horizontally from the teaser boar, which had been introduced into the farm in the same year when the increase of mummified fetuses was recorded.

Regardless of the transmission route, the fetal myocardium appears to be one of the preferred sites of viral replication, due to the vast growth of myocytes in the late stage of gestation (Beinlich et al., 1995). Although the presence of myocarditis is essential for the diagnosis of PCV2-RD (Segalés, 2012), myocarditis could not be observed in any of the fetal hearts in by histopathological examination. However, it was also demonstrated that the highest amounts of PCV2 DNA copies were mainly detected in fetal heart tissue not expressing myocarditis (Brunborg et al., 2007). Nevertheless, the absence of findings in the histopathological investigations could also be the result of decomposition of mummified piglets.

Despite the fact that lung tissue was taken from fetus 4 for quantitative PCR analyses instead of heart tissue, we were able to detect PCV2 DNA in high loads in lung tissue as well. In a study conducted by Norwegian colleagues, PCV2 DNA could be detected via PCR in several fetal organs including tissue from hearts, lungs, spleen, kidneys and brain, but heart tissue contained the highest viral loads of PCV2 (Brunborg et al., 2007). However, in the course of our investigations, lung tissue from fetus 4 yielded more positive virus signals in ISH than heart tissue. Thus, further

investigations comparing the amount of PCV2 DNA in different fetal organs could be considered.

The threshold for the amount of PCV2 DNA for the diagnosis of PCV2-RD was once defined as 10^5 PCV2 DNA copies/500 ng DNA in fetal heart tissue with myocarditis and 10^7 PCV2 copies/500 ng DNA in heart tissue without myocarditis (Brunborg et al., 2007; Hansen et al., 2010; Segalés, 2012). According to the results reported by Beinlich et al. (1995), the average weight of myocardial DNA can be estimated to be approximately 3 mg g^{-1} heart tissue (Beinlich et al., 1995). Therefore, 500 ng DNA are contained in approximately 167 µg myocardial tissue. Using this information, the threshold would be approximately 6×10^8 PCV2 DNA copies/g fetal heart tissue with myocarditis and 6×10^{10} PCV2 copies/g heart tissue without myocarditis as reported similarly before (Unterweger et al., 2021). Thus, two hearts (fetus 2 and 6) were clearly below the threshold of 10^7 PCV2 copies/500 ng DNA. This goes along with the results of ISH and other previous observations (Unterweger et al., 2021). Since weight measurement of DNA remains too laborious and costly, using a threshold defined by copies/mg tissue instead of copies/500 ng DNA should be taken into consideration. Interestingly, fetus 2 and 6, which were only weakly positive or negative for PCV2 had the best preservation status. Due to information given by different internal investigations, we assume that there could be an indirect correlation between preservation status and detectable viral load in the respective fetus.

Given that PCR results were negative for PRRSV, PPV1, EMCV, PCV3, PTV, *L. interrogans* and *Chlamydia* spp., most potential infectious causative agents of reproductive failure could be excluded or were probably not the cause of the reproductive disorders. Due to the fact that mummification has not been described to be a result of infections with influenza A virus (IAV), samples for the detection of IAV RNA were not taken. However, the presence of Japanese encephalitis virus, Aujeszky's disease (pseudorabies) virus, African and classical swine fever virus was not verified, since none of these pathogens has been detected or reported in Austria within the last ten years. Furthermore, it did not seem necessary to search for less common pathogens associated with mummification, due to the improvement of reproductive performance of the farm after the implementation of a regular PCV2 vaccination of sows and gilts.

Since the average total litter size, including mummified fetuses, was not decreased in December and February, and neither was the return to oestrus rate increased in the autumn of 2018, there is no evidence for the augmented occurrence of embryonic death within that period. This could indicate a certain resistance of embryos to infections with PCV2 in early stages of gestation (Brunborg et al., 2007). Due to the fact that measurement of embryonic death remains difficult under field conditions, most reports only refer to mummified fetuses and stillborn piglets (Brunborg et al., 2007; de Castro et al., 2012; Oropeza-Moe et al., 2017). However, a trial demonstrated a significantly decreased embryonic survival rate of embryos exposed to PCV2 (Mateusen et al., 2007). While the abortion and return to

oestrus rate remained low throughout the whole winter, the abundance of mummified fetuses accounted for approximately 15.5% in December and February but dropped to 3.5% in April, about one month after the herd vaccination, indicating that the implementation of PCV2 vaccination can further help to stabilise the sow herds (Oliver-Ferrando et al., 2018). The incidence of mummified fetuses after herd vaccination might be due to the infection of the fetuses in an early stage of gestation before the sow has been vaccinated against PCV2. Generally, vaccination of sows might be also able to inhibit a further spread of PCV2 amongst the fetuses. Throughout the rest of the year, the mummification rate remained below 3% for all farrowing groups. Due to the data we received from the farm, we suggest that an increased mummification rate is highly indicative for the occurrence of PCV2-RD.

ACKNOWLEDGEMENTS

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