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Article Title: A cohort study of post-weaning multisystemic wasting syndrome and PCV2 in 178 pigs from birth to 14 weeks on a single farm in England
Year of publication: 2010
Link to published article:
http://dx.doi.org/10.1016/j.prevetmed.2010.08.001
Publisher statement: Woodbine, K. et al. (2010). A cohort study of post-weaning multisystemic wasting syndrome and PCV2 in 178 pigs from birth to 14 weeks on a single farm in England. Preventive Veterinary Medicine, Vol. 97(2), pp. 100-106

A cohort study of post-weaning multisystemic wasting syndrome and PCV2 in 178 pigs from birth to 14 weeks on a single farm in England

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Abstract

Our hypothesis was that pigs that develop post- weaning multisystemic wasting syndrome (PMWS) are detectable from an early age with signs of weight loss and other clinical and serological abnormalities. Therefore, the objective of this study was to investigate the temporally varying and fixed events linked with the clinical incidence of PMWS by comparing affected and unaffected pigs in a cohort of 178 male piglets. Piglets were enrolled at birth and examined each week. Samples of blood were collected at regular intervals. The exposures measured were porcine circovirus type 2 (PCV2) antibody titres in all 178 and PCV2 antigen in a subset of 75 piglets. We also observed piglet health and measured their weight, and a *post mortem* examination was performed by an external laboratory on all pigs between 6 and 14 weeks of age that died. From the cohort, 14 (8%) pigs died from PMWS and 4% from other causes. A further 37 pigs between 6 and 14 weeks of age died from PMWS (30) and ileitis and other causes (7). PMWS was only apparent in pigs from 1 - 2 weeks before death when they wasted rapidly. There were no other characteristic clinical signs and no obvious gross clinical lesions *post mortem*. There was no strong link with PCV2 antibody throughout life but PCV2 antigen level was higher from 4 - 6 weeks of age in pigs that died from PMWS compared with pigs that died from other causes.

Keywords: Post-weaning multisystemic wasting syndrome, PMWS, porcine circovirus type 2, PCV2, antibody, antigen, *post mortem* examination, clinical signs, cohort

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1. Introduction

Post-weaning multisystemic wasting syndrome (PMWS) was first reported in England in late 1999 (Done et al., 2001), and has since spread throughout the United Kingdom (Woodbine et al., 2007). There are no pathognomic clinical signs of PMWS, affected pigs have some of the following signs: wasting, hairiness, enlarged lymph nodes, diarrhoea, pallor, jaundice (although none of the 330 pigs examined at *post mortem* in a cross-sectional study in GB had jaundice (Turner et al., 2008)) and dyspnoea (Harding and Clark, 1997; Chae 2004). Pathological lesions include granulomatous inflammation and the presence of intracytoplasmic inclusion bodies in the lymphoid tissue (Rosell et al., 1999) and, the most common feature, generalised depletion of lymphocytes (Nielsen et al., 2003; Darwich et al., 2004). The severity of histological lesions and viral burden vary with the stage of disease (Krakowka et al., 2005). To obtain a herd diagnosis it is recommended that several pigs are taken for *post mortem* examination (PME) to observe the full range of lesions (Harding and Clark, 1997). There is no case definition for a pig with PMWS (Turner et al., 2008).

Porcine circovirus type 2 (PCV2) is associated with PMWS (Allan and Ellis, 2000). However, the presence of PCV2 alone is not sufficient to trigger clinical signs (Segales et al., 2005) and the currently used antibody and virus tests are insufficient to define clinically affected pigs and farms (Turner et al., 2008). Larochelle et al. (2003) reported that the serological profile of PCV2 in herds from a cohort and a cross-sectional study in Quebec, with and without PMWS, were very similar, with a gradual decrease in antibody titre from 3-11 weeks of age (waning maternal immunity), seroconversion at 15 weeks of age and thereafter high titres remaining. Similarly, Blanchard et al. (2003) reported a high seroprevalence of PCV2 in sows and piglets at the end of the growth phase in 8 herds in Brittany. However, Blanchard et al. (2003) also reported that the

seroprevalence of PCV2 in 11-17 week old piglets was statistically correlated to clinical signs of PMWS. In a cohort study on 4 farms in Brittany (Rose et al. 2005) piglets with low PCV2 titres at 7 weeks of age that had not seroconverted and piglets from PCV2 negative sows were most likely to be affected with PMWS. Lopez-Soria et al. (2005) reported that pigs that were infected with PCV2 at an early age had a higher risk of developing PMWS, suggesting that the timing of infection with PCV2 is important in disease outcome.

Several farm studies have focused on risk factors for PMWS and disease progression. Susceptibility to PMWS has been reported to vary by litter (Madec et al., 2000; Allan et al., 2002; Snow and Cook, 2006; Calsamiglia et al., 2007), breed (de Jong et al., 2003; Opriessnig et al., 2006; Snow and Cook, 2006), gender (Snow and Cook, 2006) and maternal PCV2 antibody concentration (Calsamiglia et al., 2007). Madec et al. (2000) reported that the first clinical signs of PMWS were unthriftness, pallor and fever, with associated respiratory or digestive disorders, followed by wasting. Weight at weaning was not a good predictor for disease according to Madec et al. (2000), but was according to Rodriquez-Arrioja et al. (2002).

In the current paper we present a cohort study of 178 pigs where we investigate the development of PMWS. We use one farm in England with high health status to control for background disease effects on clinical and *post mortem* presentation. We focus on 178 male pigs observed from birth to death or 14 weeks of age and compare weight, clinical signs and PCV2 antibody titres and detection of PCV2 antigen over time. In addition, the PME, histology and PCV2 antigen scores from pigs that died from PWMS and those that died from other causes are compared.

2. Materials and Methods

2.1. The study farm

The study farm was initially recruited into a retrospective study of the space time patterns and risk factors associated with first signs of PMWS in June 2003 (Woodbine et al., 2007). At the time of recruitment into the retrospective study, the herd had had no increase in post-weaning mortality and according to the farm veterinarian and our case definition (Turner et al., 2008) the farm was PMWS-negative. The herd was first diagnosed with PMWS by the same farm veterinarian in March 2004. At this time, the farm was re-visited to obtain blood samples from a range of ages of pig. The farm was then recruited into the cohort study in July 2004 and visited 2-3 times a week until March 2005.

The farm was a multiplier unit, with approximately 400 sows, producing replacement gilts. It was situated 2 miles from the nearest pig unit (a small outdoor unit and, at the time of the study, PMWS-negative). The nearest PMWS-positive unit was 5 miles away. Gilts were purchased routinely at 5-6 month intervals and kept in an isolation unit approximately 400 meters from the main herd until their disease status was confirmed. The sows farrowed each week and piglets were weaned at 4 weeks of age and segregated by sex into two parts of one bungalow building. One bungalow was filled each week. At the time of the study the herd was free from porcine reproductive and respiratory syndrome (PRRS) virus. The herd had a recurrent problem with ileitis in older grower pigs.

2.2. Enrolment of pigs into the cohort

Litters that were selected to be in the study were sourced in part from sows with stored serum samples. To minimise any risk of economic loss to the multiplier farm only male piglets were recruited. Each male piglet was individually identified with an ear tag in

the first week of life. In total 178 piglets from 36 litters were enrolled into the study from birth to death or 14 weeks of age.

2.3. Data collection

Blood samples were collected from each of ten 8- and ten 14-week old pigs and five gilts, and 2, 3, 4 and ≥5 parity sows in June 2003, March 2004 and July 2004 and the serum was stored. The 178 male pigs were examined using a standard recording sheet where any deviation from normal *per se*, not from the group, was recorded by examining pigs from nose to tail. The signs observed included thin, wasting, hairiness, pallor, discharges, abnormal respiration, locomotion or nervous signs. Pigs were also weighed each week using scales or a weigh crate. Samples of blood were collected under Home Office license from pigs at approximately 4-7 days of age, before weaning (approximately 4 weeks old) and then on a bi-weekly basis until 14 weeks of age. All measurements were made by one researcher (JS).

Analysis of serum for PCV2 antibody and antigen

Blood was separated and stored at -20^{o}C at the University of Warwick, England. Sera were sent in batches to Queen's University, Belfast and analysed for antibodies to PCV2 by indirect immunoperoxidase monolayer assay (IPMA) (Rodriguez-Arrioja et al., 2000). A total of 282 serum samples from 75 pigs from 14 litters were analysed for presence of PCV2 DNA in serum. In 7 litters none of the pigs died from PMWS and in the other 7 litters 11 pigs, 1 – 4 per litter, died from PMWS. In 9 /14 litters one pig died from other causes. Over all 14 litters there were 55 pigs that survived to 10 weeks.

For the direct screen for PCV2 PCR in serum, frozen sera were rapidly thawed at 37^{o}C. 4µl of each serum sample was added to 16µl sterile PBS before heating to 95^{o}C for 3 minutes and snap cooling on ice for 5 minutes to inactivate any components of the sample that may inhibit the PCR reactions. Then 5µl of heat inactivated serum was

added to 0.5ml tubes containing 45µl of PCR mastermix (2.5 units *Taq* polymerase (Fermentas), Taq polymerase buffer (+$(NH_4)_2SO_4$ without $MgCl_2$ – Fermentas), 0.2mM each dNTP (Fermentas), 2.5mM $MgCl_2$, 20pmol each primer (ORF2PCV2S4 and ORF2PCV2AS4); After an initial denaturation step of 10 minutes at 95^{o}C, the samples were subjected to thermal cycling in a Hybaid Omn-E cycler for 40 cycles of 95^{o}C for 30 sec, 50^{o}C for 30s and 72^{o}C for 30s. The samples were then allowed a final extension at 72^{o}C for 10 minutes before running on 1.5% agarose gels containing 1mg/ml ethidium bromide and visualising under a UV transilluminator.

All serum samples from 10 pigs were subjected to DNA extraction using a QiaAmp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. After DNA extraction, PCRs were repeated as above for these samples to complement the initial screen. Positive and negative controls were included, with positive samples constructed from a known negative serum sample spiked with PCV2 DNA. These samples were reanalysed by both methods to validate the assays.

2.4. *Post mortem,* histopathology and immunohistochemical examination

The mortality data from the farm records from January 2003 to January 2006 were collected.

All pigs (male and female) that died ≤14 weeks of age from the 36 litters and all pigs on the farm aged 6 – 14 weeks were taken for *post mortem* examination (PME). All PMEs and histological examination were completed at Leeds Veterinary Laboratory (LVL) by a qualified pathologist using standard protocols (Turner et al., 2008). The tracheobronchial, ileocaecocolic and inguinal lymph nodes were collected from each pig. Half of each lymph node was submitted for histopathological examination and the other half submitted to Queen's University, Belfast and examined for PCV2 antigen level using immunohistochemistry (Krakowka et al., 2005).

PMWS was defined as present in pigs aged 6 - 14 weeks of age (Harding and Clark, 1997) that exhibited the gross clinical signs of wasting, pallor and hairiness with no other known disease (typically ileitis). The remaining pigs of this age were classified as PMWS negative.

2.5. Data analysis

Mortality data on pre-weaning, nursery, grower and finisher pigs were plotted from January 2003 to January 2006 using a two month rolling average for the participating farm. A Poisson distribution was used to test for within litter clustering of PMWS. The continuous outcomes pig weight and log PCV2 antibody titre were used to predict the change in weight and PCV2 titre by age for pigs that had clinical signs of PMWS, pigs that died from other causes and pigs that remained healthy. The models were constructed in MLwiN (version 2.1 Rasbash et al., 2000) with three hierarchical levels, observation (level 1), pig (level 2), and litter (level 3). The models included 526 measurements from 178 pigs from 36 litters. Age was included as linear and quadratic terms.

The proportion of pigs with detectable PCV2 antigen in serum was plotted for pigs that subsequently died from PMWS, other causes and survived. A logistic binomial model with 'died from PMWS' as the outcome variable with two hierarchical levels - pig (level 1) and within litter (level 2) - was used to examine associations with lesions at *post mortem*. Only the data from the 59 pigs that died between 6 and 14 weeks of age (40 from PMWS, 19 from other causes) were used in this analysis.

Results 3.1.

Farm mortality data (Figure 1)

The pre-weaning mortality percentage decreased from early 2003 to mid-November 2003, and was relatively stable in 2004, at approximately 7%. In April 2004, when PMWS was first diagnosed, the nursery pig death rate increased, with peaks in October 2004 and March 2005. In late April / early May 2004 the death rate in grower pigs increased rapidly, peaking at 5% in October 2004. This increase corresponded with an increase in nursery and finisher pig mortality rates. The death rate in growers then decreased to 2-4% in early 2005. The death rate of finishers increased slowly from November/December 2003 and continued to rise slowly in 2004 to approximately 1.5% but there was no large increase in finisher rate mortality during the study period.

3.2. PMWS distribution within litters

The mean number of male pigs that died from PMWS per litter was 0.4 (95% CI, 0.23 - 0.69). The expected distribution for 0, 1, 2, 3 and 4 deaths from PMWS per litter was 24, 10, 2, 0, and 0. The observed distribution (25, 9, 1, 0 and 1) was not significantly different ($P>0.05$).

3.3. Pig weight

None of the cohort pigs that died were weighed after 10 weeks of age. There was no significant difference in weight between pigs that died from PMWS and healthy pigs until 2 weeks before the PMWS affected pigs died. Pigs that died from any cause had a lower mean weight at death with a mean difference of 3 - 5kg compared with those that survived. There was no significant difference in weight between pigs that died from PMWS and pigs that died from other causes, indicating that wasting was different from weight loss.

3.4. Porcine circovirus type 2 antibody and antigen (Figure 2)

The PCV2 antibody titre varied by age group and visit; at the first visit in June 2003, 8-week old pigs had low antibody titres, titres were higher in 14-week old pigs and lower in gilts. At the second visit in March 2004 the PCV2 antibody titres were notably higher in 8 week old pigs and gilts than at the previous visit and were visibly higher across all ages. At the third visit in July 2004 the titres were high in 8 week old pigs and lower in 14 week old pigs. There was no significant difference between the serological antibody titres in the sows sampled at different time points. There was no association between sow antibody titre and their own piglets' neonatal antibody titres (Turner 2008).

The PCV2 antibody titres decreased in all cohort pigs from birth to 4-5 weeks of age and then increased. Pigs that died from PMWS had non-significantly lower antibody titres throughout life compared with healthy pigs or those that died from other causes and as pigs got older the difference in log PCV2 antibody titre between PMWS pigs and healthy pigs increased. This difference was never statistically significant, however, there was no difference in porcine parvovirus (PPV) antibody titres in these three groups of pigs (data not shown) indicating that this was not a generally lower antibody level in pigs that died from PMWS.

PCV2 antigen was not detected in pig serum until pigs were >28 days. PCV2 antigen was detected in up to 30% of samples. All samples from pigs that died from PMWS were antigen positive at 56 days. PCV2 antigen was significantly more likely to be present in pigs that died from PMWS compared with those that died from other causes and those that survived (OR 4.5 (1.1 – 18.2). Analysis of samples from 10 pigs using both assays indicated that there were no false positives from the initial screen but that the screen might have underestimated true positive samples. High concentrations (>2+) of PCV2 antigen

were detected in one day old dead piglets, suggesting vertical transmission. Approximately 58% of pigs that died from PMWS had PCV2 antigen levels >2+ compared with 20% of pigs that died from other causes (P = 0.02).

3.5. Clinical and *post mortem* signs (Figure 3, Table 1)

The prevalence of respiratory distress and scouring increased in the weeks preceding death from PMWS. They were significantly more prevalent in pigs one week before they died from PMWS and rare events in pigs that did not develop PMWS. Wasting and scouring were significantly associated with death from PMWS (P<0.01) when observed within 7 days of death. Respiratory distress was correlated with wasting and scouring was correlated with thin and hairy.

In total, 81 pigs were taken for PME, 59 were aged between 6-14 weeks old, 22 were in the cohort study and a total of 44 died from PMWS. In the multivariable model of all 59 pigs tail biting, slight amount of pleural fluid, presence of interlobular oedema, congestion of caudal lobes in the lungs and haemorrhage of the distal ileum were less likely to be present in pigs that died from PMWS compared with other causes. Congestion of the ileocaecocolic lymph node was present in 50% of pigs with PMWS and 87% of pigs that died from other causes (P=0.04, 95% CI, 0.010.89). This lesion did not remain significant when added to the multivariable model of gross PME abnormalities.

4. Discussion

This was a cohort study of 178 pigs on one herd with good biosecurity and good health status. The absence of porcine reproductive and respiratory syndrome virus (PRRSV) was useful to reduce confusion between causes of death and synergistic links between PRRSV and PMWS (Woodbine et al., 2007). The study was intensive with a large

amount of clinical and laboratory data produced. There were 44 pigs that died from PMWS, possibly sufficient to study associations between PMWS and clinical signs in life and *post mortem*, particularly because of the repeated observations over time and the limited background variability because we used one farm. The key results were that pigs that died from PMWS were not clinically distinguishable from other pigs until 1 - 2 weeks before they died when they were significantly more wasted; a sign specific to pigs with PMWS, and lighter in weight, also observed in pigs that died from other causes. A change in weight was not detectable at weaning in pigs that subsequently died from PMWS also reported by Madec et al. (2000) but not by Rodriquez-Arrioja et al. (2002). This suggests that the clinical development of PMWS is rapid. Wasting, which was correlated to weight but not identical to it, was more prevalent in pigs that died from PMWS than other causes and was correlated with pallor and hairiness and these signs together might indicate anaemia with loss of fat and muscle. Anaemia would cause respiratory distress in very severe cases and increases the risk of many other diseases because of debilitation. Unfortunately this rapid wasting is not itself pathognomic and so does not assist with developing a case definition of PMWS in individual pigs; however, it might help in understanding the rapid pathology caused by the disease.

Gross internal lesions *post mortem* were also not specific for PMWS and it was absence of gross lesions that was the key feature in this study. This herd had ileitis and so many of the pigs that died from other causes would have had ileitis, consequently the lesions in pigs that died from other causes were primarily in the alimentary tract. As in our previous study (Turner, 2008) no pigs with PMWS had jaundice.

There was a trend that PCV2 antibody titres were lower throughout life in pigs that developed PMWS than in those that remained healthy. This was also reported by

Meerts et al. (2006) but not Larochelle et al. (2003). Therefore, PCV2 antibody titre was not informative for future development of PMWS. There was no association between sow antibody titre and mean litter antibody titre at <1 week of age (Turner et al., 2008), suggesting that sow serum PCV2 antibody titres are not correlated to the litter serum PCV2 antibody titres. Rodriguez- Arrioja et al. (2002) also failed to detect an association between sow and PCV2 antibody titres in 3 week old piglets. One explanation for this lack of association is that PCV2 virus detected in one day old dead piglets might bind PCV2 antibody sourced from colostrum so that it is not detectable. Interestingly, pigs that died from PMWS started to have PCV2 DNA detectable in serum from 28 days of age, with an increasing proportion positive to 100% of those pigs tested that died from PMWS positive by 7-8 weeks of age. This pattern of detection of PCV2 DNA was markedly different from that in pigs that died from other causes and pigs that survived where there was a low peak in PCV2 DNA detected at about 56 days of age. This and the high viral loads in lymph nodes at *post mortem* do suggest that pigs that died from PMWS had circulating virus before death as reported by Grau-Roma et al., (2009).

Although PCV2 antibody was clustered by litter there was no clustering of deaths caused by PMWS within litters as reported by Calsamiglia et al. (2007) and Madec et al. (2000) who postulated that this might indicate genetic or maternal susceptibility. This might be a power effect because of the size of the current study, however, even if 127 litters had been studied as in Madec et al. (2000) there would have been no significant clustering of PMWS within litters if the same distribution of deaths by litters is assumed. A very large number of litters would have had to be studied to detect such an effect, suggesting that in this farm clustering was probably not occurring, one explanation for this is that the force of infection was high and that all piglets from all litters were equally exposed.

The PCV2 antibody titres in 8 week old pigs sampled at the visit in 2003 were low, suggesting that maternal antibody (if present) was lost before 8 weeks of age and that seroconversion occurred between 8 and 14 weeks of age. The 8-week old pigs were seropositive at subsequent visits suggesting that either maternal immunity was higher (at this second sampling pigs of all ages had a higher antibody titre) or that these pigs were seroconverting before 8 weeks of age. Within 4 - 8 months from when PMWS was first observed on the farm, the PCV2 antibody titres were similar to those before disease, with the exception of the 8 week old pigs. This might suggest that a rapid but short lived exposure to PCV2 occurred when PMWS was introduced that boosted herd immunity. This could have been direct, for example the introduction of a new strain of PCV2 virus (Wiederkehr et al., 2009), or indirect from introduction of another virus because stimulation of the immune system causes increased replication of PCV2 (Meerts et al., 2005). PCV2 replicates when pigs are challenged with a variety of viruses, consequently a new strain of PCV2 (as suggested by Wiederkehr et al., 2009) or another virus not yet associated with PMWS might explain the high PCV2 antigen concentration in pigs that subsequently died from PMWS. There were no other known viruses causing mortality on the farm at the time of the study.

The results from this study are specific to this farm but also control for farm effects; it is important to consider all findings in the context of previous findings as we have done above. One limitation is still the case definition for PMWS in individual pigs. There is currently no individual pig case definition (Turner et al., 2008) and the approach taken was to rule out all alternative diagnoses, exclude uncertainties and classify a pig as PMWS if it had typical clinical signs of wasting, pallor and hairiness. It is possible that the pigs classed as having died from causes other than PMWS might have had PMWS and that their abnormal clinical signs resulted from secondary disease. Similarly, pigs classed as having

PMWS might have had signs caused by another disease. No other published study to date has a more specific approach than that used here. There were no healthy pigs taken for PME for comparison unlike in Turner et al. (2008), although it is difficult to imagine that these would have been informative.

In conclusion, deaths among male pigs attributed to PMWS were not clustered within litters. Pigs with PMWS had higher PCV2 antigen in life and *post mortem* and a non-significantly lower PCV2 antibody titre throughout life. They were more likely to be lighter and wasted than pigs that did not die. These clinical signs were temporally associated with PMWS but not detectable until the week before death.

Acknowledgements This project was funded by DEFRA and BPEX/MLC. We are grateful for the active contribution of the farmer, the farm staff and the veterinarian, and a big thank you to all those that assisted in data collection and data entry.

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Figure 1. Two month rolling average mortality from January 2003 to January 2006 for pre-weaning mortality (solid line), nursery pig mortality (thick dashed line), grower mortality (light line) and finisher mortality (light dash line).

Figure 2. Mean log porcine circovirus 2 (PCV2) antibody and proportion of pigs with detectable PCV2 antigen by age in weeks

Figure 3. Time to death from PMWS and percent of pigs with clinical signs compared with those that never got PMWS

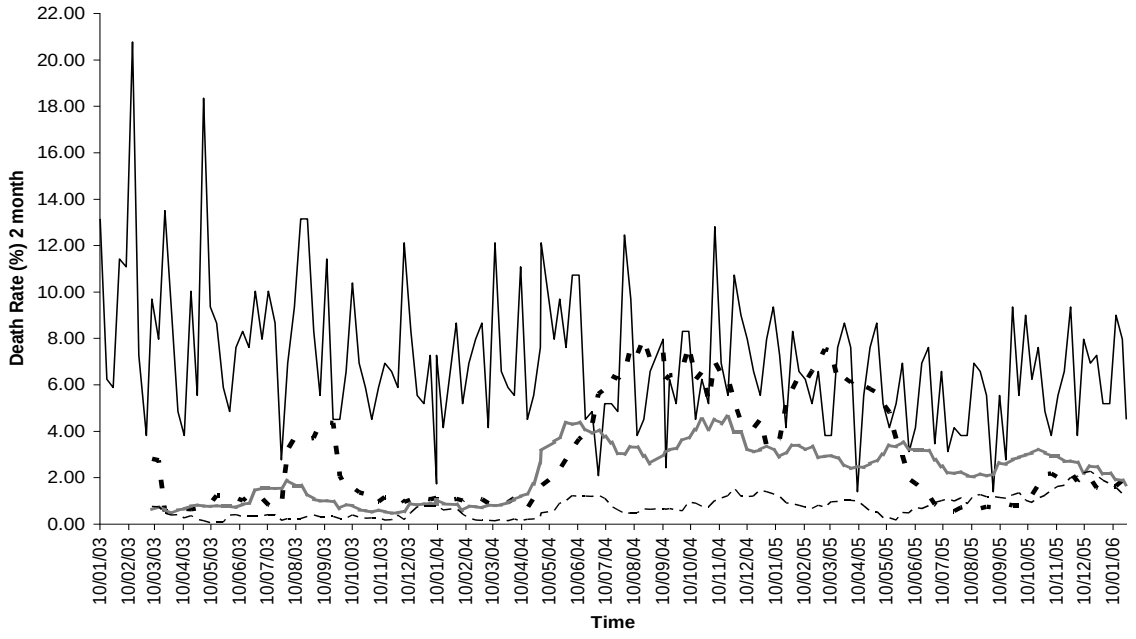

Figure 1. Two month rolling average mortality from January 2003 to January 2006 for pre-weaning mortality (solid line), nursery pig mortality (dark dotted line), grower mortality (light dotted line) and finisher mortality (light dash line).

Table 1. Multivariable model of the post mortem lesions in 59 pigs that died from post-weaning multisystemic wasting and other causes on one farm.

Variable	Died, PMWS (%)	Died, other causes (%)	Odds ratio	95% CI	P
Bitten tail	9	27	0.03	0.002,0.52	0.01
Slight amount of pleural fluid	9	27	0.05	0.003,0.60	0.02
Mild interlobular oedema in lungs	2	33	0.01	0.001,0.27	0.005
Congestion of caudal lung lobes	13	40	0.07	0.005,0.75	0.04
Haemorrhage of distal ileum	2	20	0.02	0.001,0.42	0.01

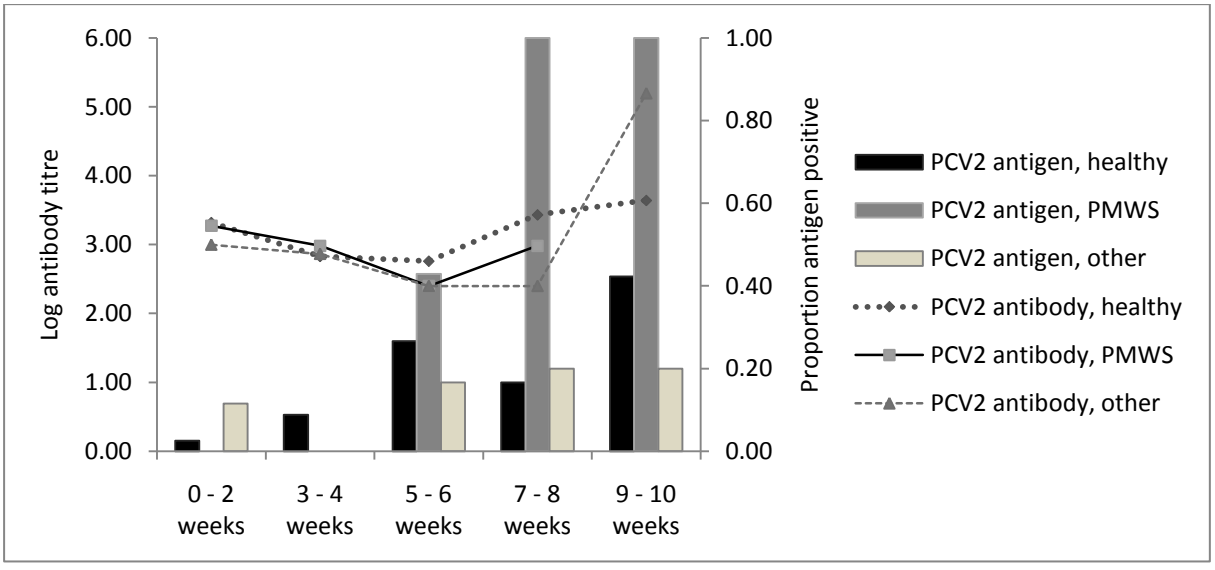

Figure 2. Mean log porcine circovirus 2 (PCV2) antibody and proportion of pigs with detectable PCV2 antigen by age in weeks

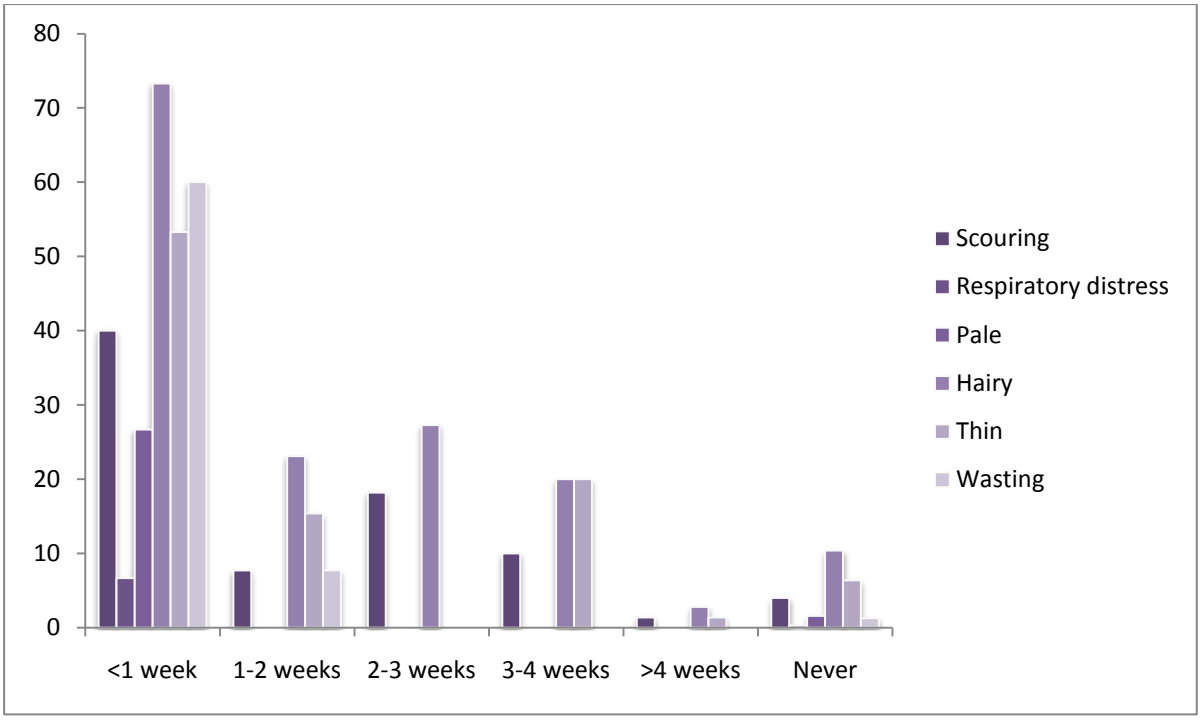

Figure 3. Time to death from PMWS and percent of pigs with clinical signs compared with those that never got PMWS