Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark

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ABSTRACT

Longitudinal case–control studies were performed in post-weaning multisystemic wasting syndrome (PMWS) affected farms from Denmark and Spain using similar designs. Fourteen independent batches of 100–154 pigs per batch were monitored from birth to PMWS outbreak occurrence. Pigs displaying PMWS-like signs and matched healthy cohorts were euthanized during the clinical outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs were classified as: (i) PMWS cases, (ii) wasted non-PMWS cases and (iii) healthy pigs. Porcine circovirus type 2 (PCV2) quantitative PCR (qPCR) and serology techniques were applied to analyse longitudinally collected sera and/or nasal and rectal swabs. Results showed that PCV2 load increased in parallel to waning maternal antibody levels, reaching the maximum viral load concurrent with development of clinical signs. PMWS affected pigs had higher PCV2 prevalence and/or viral load than healthy pigs in all collected samples at necropsy (p < 0.0001–0.05) and even in sera and nasal swabs at the sampling prior to PMWS outbreak (p = 0.01–0.05). Danish farms had a higher PCV2 prevalence in young piglets as well as an earlier PMWS presentation compared to Spanish farms. PMWS diagnoses were confirmed by laboratory tests in only half of pigs clinically suspected to suffer from PMWS. Positive and significant correlations were found among PCV2 viral loads present in sera, nasal swabs, rectal swabs and lymphoid tissues (R = 0.289–0.827, p < 0.0001–0.01), which indicates that nasal and rectal swabs were suitable indicators of PCV2 excretion. Sensitivity and/or specificity values observed from both tests used separately or combined suggested that qPCR and/or serology tests are not apparently able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis within PMWS affected farms. However, qPCR appears to be a potential reliable technique to diagnose PMWS on a population basis.

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1. Introduction

Porcine circovirus type 2 (PCV2) is considered the essential infectious agent of post-weaning multisystemic wasting syndrome (PMWS), which causes significant economic losses to the swine industry worldwide (Armstrong and Bishop, 2004). First PMWS description in Spain and Denmark were in 1997 (Segalés et al., 1997) and at the end of 2001 (Hassing et al., 2002), respectively. Afterwards, the periods of maximum number of PMWS diagnoses emitted by the two major diagnostic laboratories in both countries were 1999–2003 in Spain and 2002–2005 in Denmark (Vigre et al., 2005; Segalés et al., 2007).

PMWS most commonly affects 2–4-month-old pigs, although the disease has been also described in younger and older animals (Segalés et al., 2005a). The internationally accepted criteria to diagnose PMWS include the presence of compatible clinical signs (mainly wasting and respiratory distress), moderate to severe lymphocyte depletion with granulomatous inflammation in lymphoid tissues, and detection of moderate to high amount of PCV2 within these lesions (Segalés et al., 2005a). However, PCV2 is an ubiquitous virus that has been detected in both PMWS affected and non-affected pigs and farms by pathogen detection as well as serological methods (Larochelle et al., 2003; Rose et al., 2003; Sibila et al., 2004). Therefore, the sole detection of PCV2 antibodies in serum and/or viral DNA or antigen does not constitute PMWS diagnosis, since it only indicates evidence of PCV2 infection (Krakowka et al., 2005; Caprioli et al., 2006). However, serological and DNA detection techniques (PCR) have been widely used to monitor PCV2 infection dynamics in both PMWS affected and non-affected farms.

Most PMWS epidemiological field studies based on the above-mentioned laboratory techniques have been focused on the moment of disease outbreak. These studies have shown higher PCV2 viral load in serum, lymphoid tissues and potential shedding sites in PMWS affected pigs compared to non-affected pigs (Brunborg et al., 2004; Olvera et al., 2004; Sibila et al., 2004; Segalés et al., 2005b). Moreover, a viral load threshold to discriminate PMWS affected and non-affected pigs has been suggested (Brunborg et al., 2004; Olvera et al., 2004). In contrast, only few studies have focused on PCV2 infection dynamics, based on cross-sectional (Larochelle et al., 2003; Rose et al., 2003; Sibila et al., 2004; López-Soria et al., 2005) and longitudinal studies (Rodríguez-Arrioja et al., 2002; McIntosh et al., 2006; Carasova et al., 2007). These studies have been mainly based on serology and non-quantitative PCR techniques, and only one used quantitative PCR (qPCR) technique (Carasova et al., 2007). Overall, those studies demonstrated high levels of maternal antibodies in lactating piglets, which decreased gradually during the nursery period. PCV2 viremia usually occurred when maternal derived antibody levels waned, and decreased progressively after the increase of antibody titres (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003; Sibila et al., 2004).

Some epidemiological studies have been designed as case–control studies. Overall, higher percentages of pigs with PCV2 in serum and in nasal cavities were detected using PCR in PMWS affected farms (Sibila et al., 2004). Moreover, the earlier the PCV2 infection, the higher the risk of developing PMWS (Rose et al., 2003; López-Soria et al., 2005). However, those studies did not describe in detail the infection dynamics of PCV2 infection in PMWS affected and non-affected farms.

The aim of the present study was to describe the evolution of PCV2 infection, excretion and seroconversion in PMWS, wasted non-PMWS and healthy pigs from PMWS affected farms in two different countries, Denmark and Spain. The study period went from the first week of age until the moment of disease development.

2. Materials and methods

2.1. Study design

Two longitudinal case–control studies in PMWS affected farms, one in Spain and one in Denmark, were performed in parallel during 2005 and 2006. The diagnosis of PMWS at the farm level was confirmed according to the EU definition (www.pcvd.org/news.php) before the start of the study. Diagnostic procedures included a prevalence of pigs with wasting and mortality in nurseries plus fattening/finishing areas higher than 10%, as well as the individual diagnostic case definition fulfilment of PMWS (Segalés et al., 2005a) in at least 1 out of 5 necropsied pigs.

Production systems slightly varied between Spain and Denmark. Piglets were nursed at farrowing facilities from birth until 3 (Spain) and 4 (Denmark) weeks of age. After weaning, piglets were moved into the nursery until 8–9 (Spain) or 11–13 (Denmark), weeks of age. Finally, animals were placed in fattening areas until slaughter age.

Both studies were carried out using similar designs, and were performed on 14 independent batches of 100–154 animals from each of 8 Danish (8 batches) and 3 Spanish (6 batches) farms. Ten to 21 sows were selected in order to reach 100 piglets per batch. Number of studied farms, batches and animals, as well as specific differences between Spanish and Danish protocols, are displayed in Table 1. Studied piglets were ear-tagged at 1 week of age. Nasal and rectal swabs (Collection swab, Eurotubo, Spain; and Kirudan A/S, Denmark) as well as blood samples (Vacutainer®, Becton-Dickinson, Meylan Cedex, France) were serially collected from those piglets at established weeks of age (Table 1) and when the PMWS compatible clinical picture appeared (necropsy) (Segalés et al., 2005a). The animals were evaluated clinically at the time of samplings and the herds were visited regularly by the corresponding responsible veterinarians, in order to alert the researchers of any potential apparition of clinical signs compatible to PMWS in the studied pigs. Nasal samples were collected by swabbing deeply into the turbinates (both nostrils). Rectal swabs were obtained by introducing the swab into the rectum and swabbing the surface of the colo-rectal region. All swabs were placed in tubes containing 1 (Spain) or 2 (Denmark) ml of phosphate-buffered saline (PBS) solution. Pigs were bled at the anterior vena cava. Blood from sows were also collected at 1st sampling day by puncture in the tail.
Table 1

Experimental design of the study in Denmark and Spain.

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of farms</th>
<th>No. of batches</th>
<th>Pigs per batch</th>
<th>Sows per batch</th>
<th>Cross-fostering</th>
<th>Age of sampling prior to necropsy (weeks)</th>
<th>No. of healthy pigs necropsied per batch</th>
<th>No. of PMWS-like affected pigs necropsied</th>
<th>Tissues collecteda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>8</td>
<td>8</td>
<td>109–154</td>
<td>10</td>
<td>Yes</td>
<td>1, 4, 6, 9</td>
<td>2–5</td>
<td>4–15</td>
<td>MLN, SILN, SLN, TBLN, TONSIL</td>
</tr>
<tr>
<td>Spain</td>
<td>3</td>
<td>6</td>
<td>100–105</td>
<td>10–14</td>
<td>No</td>
<td>1, 3, 7, 11</td>
<td>3–5</td>
<td>8–19</td>
<td>MLN, SILN, SLN</td>
</tr>
</tbody>
</table>

a MLN = Mesenteric lymph node, SILN = superficial inguinal lymph node, SLN = sub-mandibular lymph node, TBLN = tracheo-bronchial lymph node.

b This range comprised 7 out of 8 studied Danish batches, but in one batch (DK-3) this number was 38.

c In 2 out of 6 Spanish studied batches (SP-3a and SP-3b) only males were followed up. Consequently, up to 21 sows were needed to select 100 pigs in those two batches.

Table 2

Characteristics of farms and studied animals. Sanitary status describes which other pathogens were known to be present or absent in the sow-farm by serological determinations at the time the study was performed. Aujeszky disease virus (ADV), porcine parvovirus (PPV), porcine reproductive and respiratory virus (PRRSV), swine influenza virus (SIV), Actinobacillus pleuropneumoniae (Ap), Lawsonia intracellularis (law), Mycoplasma hyopneumoniae (Myc), Salmonella spp. (salm), toxigenic Pasteurella multocida (PMT).

<table>
<thead>
<tr>
<th>Farm</th>
<th>Health status</th>
<th>Production system</th>
<th>Number of sows</th>
<th>Batch Weeks of age at necropsy</th>
<th>Number of necropsied pigs during PMWS outbreak</th>
<th>% Mortalitya</th>
<th>Healthy Nurseries</th>
<th>Fattening</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-1</td>
<td>PRRSV+, ADV+, Myc+, PPV+, SIV+, Salm+</td>
<td>3 sites 2400</td>
<td>SP-1 18–21</td>
<td>3 8</td>
<td>5</td>
<td>1.9</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>SP-2</td>
<td>PRRSV+, ADV–, Myc+, PPV+, SIV+, Salm+</td>
<td>2 sitesb 600</td>
<td>SP-2a 15–17</td>
<td>6 5</td>
<td>5</td>
<td>4.8</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>SP-2b</td>
<td>PRRSV+, ADV–, Myc+, PPV+, SIV+, Salm+</td>
<td>2 sitesb 600</td>
<td>SP-2b 13–15</td>
<td>3 7</td>
<td>5</td>
<td>2.9</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>SP-2c</td>
<td>PRRSV+, ADV–, Myc+, PPV+, SIV+, Salm+</td>
<td>2 sitesb 600</td>
<td>SP-2c 11–15</td>
<td>6 5</td>
<td>5</td>
<td>7.7</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>SP-3</td>
<td>PRRSV–, ADV–, Myc–, PPV+, SIV+, Salm+</td>
<td>2 sites 950</td>
<td>SP-3a 12–15</td>
<td>7 9</td>
<td>5</td>
<td>6.0</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>SP-3b</td>
<td>PRRSV–, ADV–, Myc–, PPV+, SIV+, Salm+</td>
<td>2 sites 950</td>
<td>SP-3b 12–15</td>
<td>10 9</td>
<td>5</td>
<td>1.9</td>
<td>16.8</td>
<td></td>
</tr>
</tbody>
</table>

Total number of studied animals in Spanish study: 35 43 30

Total number of studied animals in Danish study: 51 40 27

a Percentages of mortality are referred to the total number of pigs present in each phase.

b All studied 2 site farms had sites I and II located in the same farm and site III located separately.

c Not recorded data.

vessels. Once in the laboratory, blood samples were allowed to clot at 4 °C and then centrifuged at 1500 × g for 10 min at 4 °C. All samples were frozen at −80 °C until testing. When the PMWS compatible clinical picture appeared at the studied farms, healthy animals and pigs displaying PMWS-like signs were selected, euthanized and necropsied. Necropsied animals were also bled and nasal and rectal swabs taken. Piglets suffering natural death and displaying clinical signs compatible with PMWS were also selected. At necropsy, sections of lymphoid tissues were collected (Table 1) and fixed by immersion in neutral-buffered 10% formalin to assess the pathological status of both clinically healthy and diseased animals.

Characteristics of studied farms, batches and necropsied animals are displayed in Table 2. All treatments, housing, husbandry and slaughtering conditions were conformed to the European Union Guidelines and Good Clinical Practices.

2.2. Histopathology

Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two consecutive 4 μm thick sections containing collected lymphoid tissues from each pig were cut from each block. One section was processed for haematoxylin and eosin stain, while the other was for PCV2 nucleic acid detection by in situ hybridization (ISH) (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2 antigen detection (Jensen et al., 2006) for Danish cases. Four lymph nodes and tonsil (Spain) and two lymph nodes (Denmark) per pig were evaluated in a blinded fashion, by the same pathologist for three different parameters using a criteria based on previously described scoring systems (Krakowka et al., 2005; Opiressnig et al., 2006) from 0 to 4, as summarised in Table 3. The score was calculated in each lymphoid tissue as: score = (lymphoid depletion + granulomatous infiltration)/2 + PCV2 amount. PCV2 amount corresponded to the
In order to compare the two serological methods, a set of 94 serum samples were analyzed in both laboratories. A positive correlation was detected between IPMA and ELISA titres ($R = 0.84$, $p < 0.001$), and a good agreement was determined by the kappa statistic ($k = 0.688$), showing that both tests gave congruent results. It is worthy mentioning that all samples that gave negative results by IPMA were also negative by ELISA test; 6 out of 94 samples gave positive results by IPMA (low titres 1:20–1:60) and negative by ELISA.

2.4. DNA extraction and PCV2 quantitative PCR (qPCR)

DNA from Spanish samples was extracted from 200 µl of serum or 300 µl of nasal PBS solution (Nucleospin® Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) or from 300 µl of rectal PBS solution (QiAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), according to the manufacturers instructions. DNA from serum and nasal swab samples was eluted in 100 µl of elution buffer, and in 200 µl for rectal swab samples. DNA from Danish samples was extracted from 200 µl of serum or 200 µl nasal PBS solution (QiAamp DNA Mini Kit, Qiagen® GmbH, Germany) or 200 µl of rectal PBS solution (QiAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), according to the instructions of the manufacturer. DNA was eluted with 200 µl elution buffer.

PCV2 DNA was quantified using two previously described real-time qPCR techniques on Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2009) samples, respectively. The performance of both qPCRs was compared in a previous study based on field samples (Hjulsager et al., 2009). This latter work showed that the two qPCRs gave comparable results showing a highly significant linear association between results of the two assays. However, the method performed by the Danish laboratory had higher sensitivity and yielded systematically higher PCV2 load values than the one used in the Spanish laboratory (corresponding to a mean difference of 1.4 log_{10} copies of PCV2 per ml of sample).

2.5. Statistical analyses

Statsdirect (version 2.6.6, CamCode, Ashwell, UK) and SPSS 15.0 (SPSS Inc. Chicago, USA) were used for statistical analyses. Analysis of variance with Bonferroni multiple
comparisons (for normally distributed variables) and
Kruskal–Wallis and Mann–Whitney tests (for non-normally
distributed variables) were used to compare IPMA titres,
ELISA titres, lesion scores, antigen or DNA staining levels in
lymphoid tissues, and viral loads in sera, nasal swabs and
rectal swabs between the three categories of pigs studied
(PMWS cases, wasted non-PMWS cases and healthy) in both
countries. Chi-square test was used to compare the
proportions of positive qPCR results between the three
categories of pigs studied; when statistically significant
different proportions were detected, the Fischer’s exact test
was applied to compare in pairs the different categories of
pigs. The Fischer’s exact test was also applied to compare
the proportion of viremic and non-viremic sows that had at least
one PMWS case in their litter. Linear associations between
viral loads detected in the different samples, between
lesions characteristic of PMWS and amount of PCV2 in
lymphoid tissues, and between total antibody titres
detected in sows and their piglets at 1 week of age, were
determined by Pearson’s (normally distributed variables)
and Spearman’s (non-normally distributed variables) cor-
relations. To determine the diagnostic performance of
serology and qPCR techniques on clinically affected animals,
a Receiver Operating Characteristic (ROC) analysis was
carried out using StatsDirect. For this, only wasted pigs were
considered, which perfectly fits a practical situation where a
diagnosis of clinically suspected animals under field
conditions is required. Thus, PMWS cases were considered
as diseased, whereas wasted non-PMWS cases were
considered as non-diseased animals. The probability of
making a correct diagnosis was assessed through calculation
of positive predictive value (PV+) and negative predictive
value (PV−). The level of significance for all analyses (α) was
set to p < 0.05.

3. Results

3.1. Studied animals and histopathology

A total of 108 and 118 animals were necropsied and
pathologically characterized in Spain and Denmark,
respectively (Table 2). All Spanish PMWS outbreaks
occurred at the beginning of the fattening phase, whereas
all Danish clinical outbreaks were observed at nurseries.
Mean ± SD of weeks of age at necropsy were 10.6 ± 1.8
(PMWS: 10.6 ± 1.7, healthy: 10.8 ± 2.0, wasted non-PMWS:
10.3 ± 1.9) and 14.7 ± 2.5 (PMWS: 14.0 ± 2.1, healthy:
14.7 ± 2.5, wasted non-PMWS: 15.2 ± 2.7) in Denmark and
Spain, respectively. Accordingly, highest mortality ratios
were observed at nurseries (Denmark) and fattening facilities
(Spain).

After histopathological examination, 35 out of 78 (45%)
and 51 out of 91 (56%) pigs showing wasting were
diagnosed as PMWS cases in Spain and Denmark,
respectively. PMWS cases had higher amount of PCV2 in
tissues (antigen or DNA staining), as well as higher
histopathological scores for lymphoid depletion and gran-
ulomatous infiltration compared to wasted non-PMWS
cases and healthy pigs from both countries (p < 0.0001).
Moreover, wasted non-PMWS pigs had higher global
histopathological average scores (p < 0.0001), as well as
higher lymphoid depletion (p < 0.02) and granulomatous
infiltration (p < 0.01) scores, than healthy pigs from both countries. The PCV2 amount in lesions of wasted non-PMWS
pigs was higher than in healthy pigs from Spain (p < 0.01)
but not from Denmark (p = 0.5). Surprisingly, 1 (Spain) and 2
(Denmark) pigs with good clinical condition (apparently
healthy) had a global average score >4, showing both the
lesional score and the score for PCV2 amount, each above 2.
These three animals were finally not classified into the
category of healthy pigs and were consequently excluded
from the statistical analyses due to the low number of cases
with this situation.

3.2. PCV2 qPCR

Percentage of pigs with PCV2 qPCR positive results at
each sampling point from both countries are displayed in
Table 4. In Spain, results showed only sporadic positive
values before 11 weeks of age. During the last two
samplings, the number of qPCR positive pigs increased
progressively over time in all the three types of material
studied (serum and nasal and rectal swabs), reaching the
maximum levels at the moment of PMWS outbreak. In
Danish farms, 18%, 69% and 23% of sera, nasal swabs and
rectal swabs, respectively, gave positive results by PCV2
qPCR technique at the first week of age, increasing also
progressively to the moment of occurrence of clinical
disease. In Spain, a higher proportion of qPCR PCV2 positive
pigs in sera were observed among PMWS cases compared to
healthy animals at 11 weeks of age (p < 0.015), as well as in
sera (p < 0.0001), nasal (p < 0.05) and rectal swabs
(p < 0.01) at necropsy. Contrary, no statistical significant
differences were found between the prevalence of PCV2
among the three categories (PMWS, healthy and wasted
non-PMWS) of studied Danish pigs. At necropsy, PCV2
prevalence in PMWS affected pigs was 100% in all 3 types of
samples from both countries, with the exception that rectal
swabs from PMWS Spanish cases had a prevalence of only
68% (23 out of 34).

Means of PCV2 load by qPCR in positive samples (Fig. 1)
showed significant differences between PMWS cases and
healthy pigs at necropsy in serum, nasal and rectal swabs
from both countries (p < 0.001). Furthermore, it was
possible to distinguish PMWS cases from healthy pigs in
Danish farms at the sampling prior at the necropsy on sera
(p < 0.01) and nasal swabs (p < 0.05), but not in Spanish
samples, even though a tendency was observed for serum
samples (p = 0.08).

Positive and significant correlations were found in both
countries among PCV2 viral loads in sera and nasal swabs
(Spain: R = 0.693, p < 0.0001; Denmark: R = 0.663;
p < 0.001), in sera and rectal swabs (Spain: R = 0.608,
p < 0.0001; Denmark: R = 0.720, p < 0.0001) and in nasal
and rectal swabs (Spain: R = 0.593; p < 0.0001; Denmark:
R = 0.736, p < 0.0001) considering longitudinally collected
samples. Moreover, positive and significant correlations
were also found between PCV2 amount detected in tissue
by ISH/IHC and viral loads detected by qPCR in sera (Spain:
R = 0.625, p < 0.0001; Denmark: R = 0.827, p < 0.0001), in
nasal swabs (Spain: R = 0.573, p < 0.0001; Denmark:
R = 0.779, p < 0.0001) and in rectal swabs (Spain:
Table 4
Percentage of PCV2 qPCR positive serum, nasal and rectal samples at the different samplings. When statistical significant differences between studied groups were detected, percentage of qPCR positive animals per group are given in parentheses (PMWS, wasted non-PMWS and healthy pig, respectively) and differences indicated by superscript letters (a–c).

<table>
<thead>
<tr>
<th>Samplings (weeks of age in Spain–Denmark)</th>
<th>1st (1)</th>
<th>2nd (3–4)</th>
<th>3th (6–7)</th>
<th>4th (9–11)</th>
<th>5th*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spanish farms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sera</td>
<td>0</td>
<td>0.9</td>
<td>0.9</td>
<td>40.7 (54a; 46b; 21c)</td>
<td>76.0 (100a; 83b; 41c)</td>
</tr>
<tr>
<td>Nasal</td>
<td>0.9</td>
<td>2.8</td>
<td>31.5</td>
<td>70.4</td>
<td>87.0 (100a; 86b; 76d)</td>
</tr>
<tr>
<td>Rectal</td>
<td>8.4</td>
<td>1.9</td>
<td>10.3</td>
<td>45.8</td>
<td>50.5 (66a; 51b; 28b)</td>
</tr>
<tr>
<td><strong>Danish farms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sera</td>
<td>18.4</td>
<td>16.0</td>
<td>41.0</td>
<td>93.4</td>
<td>94.8</td>
</tr>
<tr>
<td>Nasal</td>
<td>69.3</td>
<td>87.5</td>
<td>96.4</td>
<td>96.4</td>
<td>100</td>
</tr>
<tr>
<td>Rectal</td>
<td>22.8</td>
<td>35.1</td>
<td>50.9</td>
<td>96.3</td>
<td>98.5</td>
</tr>
</tbody>
</table>

*5th sampling correspond to necropsy moment in both countries.

Fig. 1. Mean of positive log10(copies of PCV2 per ml of serum or per ml of swab material) in serum (A), nasal swabs (B) and rectal swabs (C) by qPCR in PMWS (■), wasted non-PMWS (▲) and healthy (●) pigs at the different samplings from Spanish (left) and Danish (right) farms. Letters (a)–(c) indicates statistical significant differences between groups ($p < 0.05$).

$R = 0.289$, $p < 0.01$; Denmark: $R = 0.507$, $p < 0.0001$) collected at the moment of necropsy from both countries.

One healthy and 2 PMWS Danish cases were qPCR positive in sera at all sampling times (from 1 to 10–11 weeks of age). These animals were positive in nasal swabs and negative in rectal swabs at the first week of age and intermittently positive in both swabs afterwards. Moreover, the only Spanish pig that was PCV2 positive by qPCR
in sera at 7 weeks of age was a PMWS case which was also PCV2 qPCR positive in sera, nasal and rectal swabs until necropsy (at 12 weeks of age).

Positive PCV2 qPCR results were found in 2 out of the 87 (2.3%) and in 20 out of 68 (29.4%) studied sow's sera from Spain and Denmark, respectively (Table 5). In Denmark, 9 out of 20 sows with low but detectable viremia (45.0%) and 18 out of 48 sows with no detectable viremia (37.5%) had one or more PMWS affected pig in their litter \( (p = 0.596) \). Thus, there was no indication of any association between sow-viremia and bearing of PMWS cases in the Danish study. Due to the low number of PCV2 positive sows in the Spanish data-set, a similar analysis based on Spanish data was not possible.

### 3.3. PCV2 serology

PCV2 serology results from both countries are displayed in Fig. 2. PCV2 maternal antibodies were present at the first week of age in all pigs from both countries. In Spain, healthy pigs had significantly higher titres than PMWS and wasted non-PMWS cases at 1 week of age \((p < 0.05)\). However, no statistical significant differences were found between antibody titres detected at 3 and 7 weeks of age. Moreover, Spanish PMWS cases had lower antibody titres than non-PMWS affected pigs (healthy and wasted non-PMWS cases) at 11 weeks of age \((p < 0.05)\) and at necropsy \((p < 0.05)\). In Denmark, healthy pigs had statistically significant higher PCV2 maternal antibody titres than PMWS cases during the first three samplings \((p < 0.01)\). No statistical significant differences were obtained in Danish pigs after seroconversion.

For the Spanish study, a significant and positive correlation was found between sow PCV2 antibody titres and PCV2 antibody titres of their piglets at 1 week of age \((R = 0.66, p < 0.0001)\). No correlation was found for antibody titers between Danish sow and piglets \((R = 0.2, p = 0.2)\).

### 3.4. Diagnostic performance of qPCR and serological tests

Serology and qPCR results from samples coming from pigs with clinical signs compatible with PMWS at necropsy were used in a ROC analysis to evaluate the diagnostic performance of those techniques for live animals and the optimal conditions under which those techniques should be applied. Optimal cut-off values for each studied sample material and technique applied, together with their sensitivity, specificity, PV+ and PV− values, are displayed in Table 6. Optimal cut-offs were calculated maximizing both sensitivity and specificity values.

None of the tests on the studied samples were specific and sensitive enough to diagnose PMWS at the level of individual pigs in any of the two countries. Consequently, none of the tests on the studied samples gave PV+ or PV− values higher than 90% in both countries. On the other hand, taking into account the previously described bias between the two qPCR assays of approximately 1.4\( \log \) copies PCV2/ml \((Hjulsager et al., 2009)\), qPCR on sera gave similar optimal cut-off for both qPCRs (6.21 in Spain and 7.43 in Denmark). This fact led us to further explore the diagnostic performance of a combination of qPCR in sera and serology tests for the Danish and the Spanish data-sets. Results showed that the serial use of qPCR from sera and serology gave adequate results in terms of specificity in both countries, but with an evident lack of sensitivity.

Thus, when Spanish qPCR serum load \(> 6.21\) and IPMA titres \(> 1:1620\) were simultaneously considered to confirm or discard a PMWS case, specificity and PV+ were 100%, but sensitivity was 42.9% and PV− was 68.3%. Regarding Danish tests, when qPCR values in sera were \(> 7.43\) and ELISA titres were 0, specificity was 93.3%, PV+ 83.3%, sensitivity 21.7% and PV− 43.8%.

### 4. Discussion

The lack of an effective and consistent experimental model to reproduce PMWS makes especially relevant to
carry out epidemiological studies within PMWS affected farms. Up to date, few longitudinal epidemiological data are available on PCV2 infection dynamics in PMWS affected farms (Rodrı´guez-Arrioja et al., 2002; McIntosh et al., 2006; Carasova et al., 2007). In the present work, serology and qPCR techniques were used to describe the evolution of PCV2 infection and excretion in histopathologically well-characterized pigs (PMWS cases, wasted non-PMWS cases and healthy pigs) from two different countries: Spain and Denmark. This is the first collaborative multi-centre epidemiological study on PMWS, which has been carried-out on different farms and in two different countries.

Overall, similar PCV2 dynamic patterns were observed between countries. Serological analyses showed maternal antibody titres in all piglets at 1 week of age, which decreased gradually and reached a minimum at approximately 6–7 weeks of age. PCV2 viral load in serum increased concurrently to waning of maternal antibody levels, reaching the highest level at PMWS outbreak in all the three studied types of samples (serum, and nasal and rectal swabs) in both countries. As expected (Brunborg et al., 2004; Olvera et al., 2004), PCV2 load was higher in PMWS affected than in healthy pigs at the moment of necropsy. Interestingly, even at the sampling previous to the clinical outcome (9 and 11 weeks of age for Denmark and Spain, respectively), PMWS cases had significant higher PCV2 load (Denmark) or PCV2 prevalence (Spain) than healthy pigs, suggesting that PMWS affected pigs suffered from a higher viral load and/or longer viremia than healthy ones.

A previous comparison of both qPCR showed that despite the results obtained by both techniques had a good correlation, the Danish assay was more sensitive and yielded systematically higher PCV2 viral loads than the Spanish assay (Hjulsager et al., 2009). However, these differences in qPCR performance cannot explain all the variation between the findings in Spain and Denmark. Additional variation in results potentially could be due to the employment of different extraction methods, which could also affect the sensitivity of PCV2 detection (Fahle and Fischer, 2000; Cler et al., 2006).

PMWS was described as enzootic and epizootic presentations during the studied period in Spain and Denmark, respectively (Segalés et al., 2007). Interestingly, the high percentage of nasal Danish swabs positive by qPCR at younger ages agrees with a previous work performed during the years of maximum PMWS affection (epizootic presentation) in Spain, which was based on conventional non-quantitative PCR on nasal swabs (Sibila et al., 2004). Furthermore, present results showed a substantial delay in PMWS age-presentation in Spain compared to Denmark. These data suggested the existence of a relation between early infection and age at disease presentation. The apparent higher PCV2 prevalence observed in young Danish piglets and sows suggested that an earlier and

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![Mean + SD coded titres for porcine circovirus type 2 (PCV2) total antibodies in PMWS (---), wasted non-PMWS (-----) and healthy (-----) pigs at the different samplings from Spanish (above) and Danish (below) farms. Different letters (a–c) in each sampling indicate significant differences between groups \( p < 0.05 \).](image)

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### Table 6

Optimal cut-off values, sensitivity, specificity, positive predictive value (PV+) and negative predictive value (PV−) obtained from ROC analyses from serological and quantitative PCR (qPCR) techniques in each of studied samples. qPCR results are expressed as log_{10} copies of PCV2 per ml sample.

<table>
<thead>
<tr>
<th>Country</th>
<th>Sample</th>
<th>Test</th>
<th>Optimal cut-off</th>
<th>Sensitivity (CI 95%)</th>
<th>Specificity (CI 95%)</th>
<th>PV+ (CI 95%)</th>
<th>PV− (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>Nasal swab</td>
<td>qPCR</td>
<td>≥5.9</td>
<td>68.6 (31.4–66.0)</td>
<td>74.4 (77.8–97.4)</td>
<td>68.6 (52.5–86.9)</td>
<td>74.4 (67.5–90.4)</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>qPCR</td>
<td>≥5.9</td>
<td>41.2 (24.7–59.3)</td>
<td>97.7 (87.7–99.9)</td>
<td>93.3 (68.1–99.8)</td>
<td>67.7 (55.3–80.2)</td>
</tr>
<tr>
<td></td>
<td>Sera</td>
<td>qPCR</td>
<td>≥6.21</td>
<td>48.6 (31.4–66.0)</td>
<td>90.7 (77.8–97.4)</td>
<td>81.0 (58.1–94.6)</td>
<td>68.4 (55.5–81.4)</td>
</tr>
<tr>
<td></td>
<td>IPMA</td>
<td>≤1:1620</td>
<td></td>
<td>73.5 (55.6–87.1)</td>
<td>88.4 (74.9–96.1)</td>
<td>83.3 (65.3–94.4)</td>
<td>80.9 (68.5–93.2)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Nasal swab</td>
<td>qPCR</td>
<td>≥9.2</td>
<td>86.2 (68.3–96.1)</td>
<td>81.3 (54.4–96.0)</td>
<td>89.3 (71.8–97.7)</td>
<td>76.4 (50.1–93.2)</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>qPCR</td>
<td>≥8.1</td>
<td>50.3 (31.3–68.7)</td>
<td>82.4 (56.6–96.2)</td>
<td>83.3 (58.6–96.4)</td>
<td>48.3 (28.4–68.2)</td>
</tr>
<tr>
<td></td>
<td>Sera</td>
<td>qPCR</td>
<td>≥7.43</td>
<td>91.3 (72.0–98.9)</td>
<td>46.7 (21.3–73.4)</td>
<td>72.4 (54.4–90.4)</td>
<td>77.8 (40.0–97.2)</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>0</td>
<td></td>
<td>92.9 (76.5–99.1)</td>
<td>27.8 (9.7–53.3)</td>
<td>66.7 (50.6–82.7)</td>
<td>71.4 (29.0–96.3)</td>
</tr>
</tbody>
</table>
higher PCV2 infection pressure was present in Danish farms compared to Spanish ones, which might be the reason for an earlier PMWS presentation and reflects the epidemiological disease presentation in both countries during the studied period.

The positive and significant correlation found between amounts of PCV2 detected in lymphoid tissues, sera and swabs suggests that nasal and rectal swabs are suitable indicators of the level of PCV2 excretion. However, it has to be taken into account that swab samples may be associated with a certain degree of error if results from swab samples collected in different ways are being compared, which is not the case in the present study. Significant differences in viral loads in rectal and nasal swabs between PMWS affected and healthy animals support the idea that pigs suffering from PMWS excrete higher levels of PCV2 by nasal and rectal routes when showing the disease (Segalés et al., 2005b). These differences were also observed at the sampling prior to the clinical manifestation, when higher viral load in PMWS affected pigs was detected in Danish nasal swabs. Results also showed that a higher prevalence of PCV2 was present in the nasal cavity than in faeces during the PMWS outbreak, further supporting the oro-nasal route as the most important route of PCV2 infection and transmission (Segalés et al., 2005a).

The results of the present study indicated that the major spread of PCV2 occurred between 4 and 6 weeks of age in Danish farms and between 7 and 11 weeks of age in Spanish farms, which corresponded to the nursery phase (Denmark) and/or at the beginning of fattening (Spain). However, the fact that some sows were viremic during the lactating period together with PCV2 detection in piglets demonstrated that PCV2 infection was already present at pre-weaning ages and support the possible transmission of virus from sows to nursing piglets (Larochelle et al., 2003; Sibila et al., 2004) and also points out a potential vertical transmission (Pensaert et al., 2004; Shibata et al., 2006). In fact, PCV2 DNA was repeatedly found in sera of pigs from 7 to 70 days of life, further supporting the idea that some animals can be persistently infected under field conditions, even in the presence of high levels of PCV2 specific antibodies (Rodríguez-Arrioja et al., 2002; McIntosh et al., 2006). Those viremic pigs were demonstrated to intermittently excrete PCV2 in nasal excretions and faeces, which could potentially cause infection to their littermates and contribute to virus dissemination in weaning and/or fattening facilities. On the other hand, no difference between PMWS occurrence in piglets from viremic and non-viremic Danish sows was found, which is in contradiction to a previously published field study (Calsamiglia et al., 2007), probably due to cross-fostering practices which were allowed in Danish farms, in contrast to the above-mentioned study.

PMWS cases had lower maternal antibody titres than healthy ones in both studied countries, supporting previous studies indicating that total antibody titres may confer a certain protection against PCV2 infection and clinical disease (Rodríguez-Arrioja et al., 2002; López-Soria et al., 2005; Calsamiglia et al., 2007). Maternal antibodies at the first week of age were correlated with sow antibody levels in the Spanish study, but not in the Danish one, probably as a consequence of cross-fostering practice in the Danish study. Antibody titres after infection in Spanish pigs showed that non-PMWS affected animals (both healthy and wasted non-PMWS affected pigs) had a higher antibody response, being able to produce higher antibody titres than PMWS affected animals. The fact that Spanish wasted non-PMWS and healthy pigs were as a mean 2 and 1 week, respectively, older than PMWS could contribute in explaining these differences at the moment of necropsy, but not at 11 weeks of age. It is possible that wasted non-PMWS affected animals, although infected with higher PCV2 loads than healthy pigs, were able to overcome or limit the disease expression by the development of PCV2 antibodies, among other potential factors. However, these differences were not observed in Danish farms. Taken together, these findings suggest that total antibodies are not the only factor involved in avoiding PMWS development (Meerts et al., 2006; Fort et al., 2007). Other factors such as levels of neutralizing antibodies or cellular immunity, although they were not evaluated in the present work, were previously demonstrated to play an important role in PMWS development (Segalés et al., 2004; Fort et al., 2007).

Histopathological results showed that only half of pigs suspected to suffer from PMWS in an affected farm during the acute outbreak corresponded to true PMWS affected animals, according to the internationally accepted individual case definition (Segalés et al., 2005a). There is a wide range of causes apart from PMWS that can produce wasting in pigs (Harding, 1997), which makes the group of wasted non-PMWS affected pigs difficult to interpret collectively from a diagnostic point of view. Severity of PMWS-like lesions and amount of PCV2 in lymphoid tissues were evaluated by the above-described average histopathological score, which is based on scores for each histopathological finding, each varying from 0 to 4. Thus, wasted non-PMWS pigs with a global average score close to 0 might correspond to animals suffering from wasting due to PMWS-unrelated causes such as other infectious or non-infectious diseases and/or management conditions. On the contrary, wasted non-PMWS pigs with global scoring close to 4 might correspond to convalescent pigs or to pigs that were able to overcome or limit the disease expression (Segalés, 2002; Krakowka et al., 2005). The fact that wasted non-PMWS affected pigs had higher global histopathological scores than healthy animals, suggests that PCV2 was involved to some degree in the clinical signs that those pigs displayed.

Most healthy pigs had practically no PMWS-like lesions and none to low amounts of PCV2 in lymphoid tissues. However, approximately one third of Spanish and Danish healthy pigs had global histopathological scores $\geq 2$. This finding indicated that characteristic mild PMWS microscopic lesions can be present in subclinically PCV2 infected pigs in the field, which has been previously observed in both experimental (Allan et al., 1999; Grasland et al., 2005; Krakowka et al., 2007) and field conditions (Quintana et al., 2001). Surprisingly, 3 out of the 57 euthanized clinically healthy animals (one from Spain and two from Denmark) had moderate to severe lesions and moderate to high amount of PCV2 in lymphoid tissues. These three animals...
had also high amount of PCV2 in sera, nasal and rectal swabs. One possible explanation for this situation would be that these animals were in the initial phase of PMWS development (Segalés, 2002), thus displaying minimal or no signs of disease yet. On the other hand, those three animals had moderate to high PCV2 antibody titres, which alternatively suggests that antibody response was maybe enough to counteract the potential detrimental effect of the infection (overt clinical disease). It has to be taken into account, however, that the criterion to select healthy animals was the clinical evaluation at the moment of PMWS outbreak, selecting the best pigs from a reduced group comprised by 100 pigs ear-tagged at 1 week of age. Therefore, pre-clinical signs such as initial decrease of growing or loose of weight could have been unnoticed. For obvious reasons, it is not possible to assess if these 3 animals would have been clinically affected by the disease subsequently.

Diagnostic performance of both qPCR and serology tests used in Denmark and Spain was assessed to evaluate if they can be useful to diagnose PMWS in live animals. This was especially relevant in the present work because most of samples were obtained from live animals, fitting perfectly a practical situation in which veterinarians may be able to diagnose PMWS without having to euthanize suspected animals. Taking into account the bias among the qPCR methods used (Hjulsager et al., 2009), sera was the only type of sample that gave an optimal cut-off value similar in both countries. Those values were close to the threshold of 10^7 PCV2 copies per ml of sera previously proposed (Brunborg et al., 2004; Olvera et al., 2004; Fort et al., 2007). Differences in sampling procedure and material used could explained the different optimal cut-offs values obtained by nasal and rectal swabs in both countries. Overall, our results indicated that PCV2 qPCR (mainly from sera and nasal swabs) can give valuable information related to PMWS status in a group of pigs, being able to differentiate a population of PMWS affected from one of non-PMWS affected pigs during a PMWS outbreak and even some days before. However, a desirable probability of making a correct individual diagnosis higher than 90% was far from being achieved. The combination of the qPCR technique and a serology test improved specificity and PV+, indicating that such combination could be used to potentially confirm PMWS in live PMWS-suspected animals, but with a high percentage of false negatives due to their low combined sensitivity and PV—. Therefore, the lack of sensitivity and/or specificity values observed from both tests used separately or combined suggested that qPCR and/or serology tests are not able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis within PMWS affected farms.

The present report is the first descriptive infection dynamics study performed in multiple PMWS affected farms from different countries. This work describes the evolution of PCV2 infection, excretion and PCV2 antibodies in histopathologically well-characterized pigs. Vaccines against PCV2 have been recently developed and are under commercial use in different parts of the world. Understanding PCV2 infection dynamics in the field is considered crucial for a proper design of vaccine strategies and its corresponding monitoring.

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References


