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1 **Infection, excretion and seroconversion dynamics of porcine circovirus type 2**
2 **(PCV2) in pigs from postweaning multisystemic wasting syndrome (PMWS)**
3 **affected farms in Spain and Denmark**

4
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24

25

26 **Abstract**

27 Longitudinal case-control studies were performed in post-weaning multisystemic
28 wasting syndrome (PMWS) affected farms from Denmark and Spain using similar
29 designs. Fourteen independent batches of 100 to 154 pigs per batch were monitored
30 from birth to PMWS outbreak occurrence. Pigs displaying PMWS-like signs and
31 matched healthy cohorts were euthanized during the clinical outbreak. PMWS was
32 diagnosed according to internationally accepted criteria and pigs were classified as: i)
33 PMWS cases, ii) wasted non-PMWS cases and iii) healthy pigs. Porcine circovirus type
34 2 (PCV2) quantitative PCR (qPCR) and serology techniques were applied to analyse
35 longitudinally collected sera and/or nasal and rectal swabs. Results showed that PCV2
36 load increased in parallel to waning maternal antibody levels, reaching the maximum
37 viral load concurrent with development of clinical signs. PMWS affected pigs had
38 higher PCV2 prevalence and/or viral load than healthy pigs in all collected samples at
39 necropsy ($p < 0.0001$ to 0.05) and even in sera and nasal swabs at the sampling prior to
40 PMWS outbreak ($p < 0.01$ to 0.05). Danish farms had a higher PCV2 prevalence in
41 young piglets as well as an earlier PMWS presentation compared to Spanish farms.
42 PMWS diagnoses were confirmed by laboratory tests in only half of pigs clinically
43 suspected to suffer from PMWS. Positive and significant correlations were found
44 among PCV2 viral loads present in sera, nasal swabs, rectal swabs and lymphoid tissues
45 ($R = 0.289$ to 0.827 , $p < 0.0001$ to 0.01), which indicates that nasal and rectal swabs were
46 suitable indicators of PCV2 excretion. Sensitivity and/or specificity values observed
47 from both tests used separately or combined suggested that qPCR and/or serology tests
48 are not apparently able to substitute histopathology plus detection of PCV2 in tissues for
49 the individual PMWS diagnosis within PMWS affected farms. However, qPCR appears
50 to be a potential reliable technique to diagnose PMWS on a population basis.

51

52 **Keywords:** porcine circovirus type 2 (PCV2); postweaning multisystemic wasting
53 syndrome (PMWS); epidemiology; quantitative real-time PCR (qPCR); longitudinal
54 case-control study

55

56 **1. Introduction**

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

65

66 PMWS most commonly affects 2-4 month-old pigs, although the disease has been also
67 described in younger and older animals (Segalés et al., 2005a). The internationally
68 accepted criteria to diagnose PMWS include the presence of compatible clinical signs
69 (mainly wasting and respiratory distress), moderate to severe lymphocyte depletion with
70 granulomatous inflammation in lymphoid tissues, and detection of moderate to high
71 amount of PCV2 within these lesions (Segalés et al., 2005a). However, PCV2 is an
72 ubiquitous virus that has been detected in both PMWS affected and non-affected pigs
73 and farms by pathogen detection as well as serological methods (Larochelle et al., 2003;
74 Rose et al., 2003; Sibila et al., 2004). Therefore, the sole detection of PCV2 antibodies
75 in serum and/or viral DNA or antigen of the virus does not constitute PMWS diagnosis,

76 since it only indicates evidence of PCV2 infection (Krakowka et al., 2005; Caprioli et
77 al., 2006). However, serological and DNA detection techniques (PCR) have been
78 widely used to monitor PCV2 infection dynamics in both PMWS affected and non-
79 affected farms.

80

81 Most PMWS epidemiological field studies based on the abovementioned laboratory
82 techniques have been focused on the moment of disease outbreak. These studies have
83 shown higher PCV2 viral load in serum, lymphoid tissues and potential shedding sites
84 in PMWS affected pigs compared to non-affected pigs (Brunborg et al., 2004; Olvera et
85 al., 2004; Sibila et al., 2004; Segalés et al., 2005b). Moreover, a viral load threshold to
86 discriminate PMWS affected and non-affected pigs has been suggested (Brunborg et al.,
87 2004; Olvera et al., 2004). In contrast, only few studies have focused on PCV2 infection
88 dynamics, based on cross-sectional (Larochelle et al., 2003; Rose et al., 2003; Sibila et
89 al., 2004; Lopez-Soria et al., 2005) and longitudinal studies (Rodriguez-Arrijoja et al.,
90 2002; McIntosh et al., 2006; Carasova et al., 2007). These studies have been mainly
91 based on serology and non-quantitative PCR techniques, and only one used quantitative
92 PCR (qPCR) technique (Carasova et al., 2007). Overall, those studies demonstrated
93 high levels of maternal antibodies in lactating piglets, which decreased gradually during
94 the nursery period. PCV2 viremia usually occurred when maternal derived antibody
95 levels waned, and decreased progressively after the increase of antibody titres
96 (Rodriguez-Arrijoja et al., 2002; Larochelle et al., 2003; Sibila et al., 2004).

97

98 Some epidemiological studies have been designed as case-control studies. Overall,
99 higher percentages of pigs with PCV2 in serum and in nasal cavities were detected
100 using PCR in PMWS affected farms (Sibila et al., 2004). Moreover, the earlier the

101 PCV2 infection, the higher the risk of developing PMWS (Rose et al., 2003; Lopez-
102 Soria et al., 2005). However, those studies did not describe in detail the infection
103 dynamics of PCV2 infection in PMWS affected and non-affected farms.

104

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

109

110 **2. Materials and methods**

111 *2.1. Study design*

112 Two longitudinal case-control studies in PMWS affected farms, one in Spain and one in
113 Denmark, were performed in parallel during 2005 and 2006. The diagnosis of PMWS at
114 the farm level was confirmed according to the EU definition (www.pcvd.org/news.php)
115 before the start of the study. Diagnostic procedures included a prevalence of pigs with
116 wasting and mortality in nurseries plus fattening/finishing areas higher than 10%, as
117 well as the individual diagnostic case definition fulfilment of PMWS (Segalés et al.,
118 2005a) in at least 1 out of 5 necropsied pigs.

119

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

124

125 Both studies were carried out using similar designs, and were performed on 14
126 independent batches of 100 to 154 animals from each of 8 Danish (8 batches) and 3
127 Spanish (6 batches) farms. Ten to 21 sows were selected in order to reach 100 piglets
128 per batch. Number of studied farms, batches and animals, as well as specific differences
129 between Spanish and Danish protocols, are displayed in table 1. Studied piglets were
130 ear-tagged at 1 week of age. Nasal and rectal swabs (Collection swab, Eurotubo, Spain;
131 and Kirudan A/S, Denmark) as well as blood samples (Vacutainer®, Becton-Dickinson,
132 Meylan Cedex, France) were serially collected from those piglets at established weeks
133 of age (table 1) and when the PMWS compatible clinical picture appeared (necropsy)
134 (Segalés and Domingo, 2002). The animals were evaluated clinically at the time of
135 samplings and the herds were visited regularly by the corresponding responsible
136 veterinarians, in order to alert the researchers of any potential apparition of clinical
137 signs compatible to PMWS in the studied pigs. Nasal samples were collected by
138 swabbing deeply into the turbinates (both nostrils). Rectal swabs were obtained by
139 introducing the swab into the rectum and swabbing the surface of the colo-rectal region.
140 All swabs were placed in tubes containing 1 (Spain) or 2 (Denmark) ml of phosphate-
141 buffered saline (PBS) solution. Pigs were bled at the anterior vena cava. Blood from
142 sows were also collected at 1st sampling day by puncture in the tail vessels. Once in the
143 laboratory, blood samples were allowed to clot at 4°C and then centrifuged at 200 x g
144 for 10 minutes at 4°C. All samples were frozen at -80°C until testing. When the PMWS
145 compatible clinical picture appeared at the studied farms, healthy animals and pigs
146 displaying PMWS-like signs were selected, euthanized and necropsied. Necropsied
147 animals were also bled and nasal and rectal swabs taken. Piglets suffering natural death
148 and displaying clinical signs compatible with PMWS were also selected. At necropsy,
149 sections of lymphoid tissues were collected (table 1) and fixed by immersion in neutral-

150 buffered 10% formalin to assess the pathological status of both clinically healthy and
151 diseased animals.

152

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

156

157 *2.2. Histopathology*

158 Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two
159 consecutive 4 µm thick sections containing collected lymphoid tissues from each pig
160 were cut from each block. One section was processed for haematoxylin and eosin stain,
161 while the other was for PCV2 nucleic acid detection by in situ hybridization (ISH)
162 (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2
163 antigen detection (Jensen et al., 2006) for Danish cases. Four lymph nodes and tonsil
164 (Spain) and two lymph nodes (Denmark) per pig were evaluated in a blinded fashion, by
165 the same pathologist for three different parameters using a criteria based on previously
166 described scoring systems (Krakowka et al., 2005; Opriessnig et al., 2006) from 0 to 4,
167 as summarised in table 3. The score was calculated in each lymphoid tissue as: Score =
168 (lymphoid depletion + granulomatous infiltration)/2 + PCV2 amount. PCV2 amount
169 corresponded to the level of PCV2 DNA staining (ISH) or PCV2 antigen (IHC) detected
170 in the lymphoid tissue (table 3). Afterwards, a global average histopathological score
171 per pig was calculated by summing the individual scores of all five or two lymphoid
172 tissues and divided by five or two, respectively. Global average scores ranged from 0 to
173 8. A pig was diagnosed as PMWS when it suffered clinical signs compatible with
174 PMWS (mainly wasting) and the global average score was superior to 4. All the pigs

175 classified as PMWS cases had to had an amount of PCV2 antigen/nucleic acid of 2 or
176 more in at least one studied lymphoid tissue. Based on this global scoring system and
177 the clinical condition, necropsied pigs were classified into three different categories
178 (Grau-Roma et al., 2008): i) PMWS cases: pigs showing clinical wasting and a global
179 scoring >4 , corresponding to pigs with moderate to severe lymphoid lesions and
180 moderate to high amount of PCV2 antigen/nucleic acid; ii) Wasted non-PMWS cases:
181 pigs showing clinical wasting but a global scoring ≤ 4 , corresponding to no or slight
182 PMWS characteristic histopathological lesions and no or low amount of PCV2
183 antigen/nucleic acid within lymphoid tissues; iii) Healthy pigs: pigs showing good
184 clinical condition, which presented low global scoring averages (≤ 4), corresponding to
185 no or slight PMWS characteristic histopathological lesions and no or low amount of
186 PCV2 antigen/nucleic acid within lymphoid tissues.

187

188 *2.3. PCV2 Serology*

189 Collected sera were analyzed by serology to detect individual antibody titers to PCV2.
190 An immunoperoxidase monolayer assay (IPMA) (Rodriguez-Arriola et al., 2000) was
191 applied on Spanish samples using serial three-fold dilution of sera from 1:20 to 1:4860.
192 An in-house Enzyme Linked Immunosorbent Assay (ELISA) technique (Enøe et al.,
193 2006) was used to analyze the Danish samples. All serologic titers were expressed as
194 reciprocal dilutions and coded titers corresponding to \log_{10} values in order to facilitate
195 statistical analysis and graphic representations. Thus, the equivalence used between
196 serologic titers and coded titers in IPMA was: 0=seronegative, 1.3 = 1:20, 1.8 = 1:60,
197 2.3 = 1:180, 2.7 = 1:540, 3.2 = 1:1620 and 3.7 = 1:4860. The equivalence in ELISA test
198 was: 0=seronegative, 1.0=1:10, 1.7=1:50, 2.4=1:250, 3.1=1:1250, 3.8=1:6250 and
199 4.5=1:31250.

200

201 In order to compare the two serological methods, a set of 94 serum samples were
202 analyzed in both laboratories. A positive correlation was detected between IPMA and
203 ELISA titres ($R=0.84$, $p<0.001$), and a good agreement was determined by the kappa
204 statistic ($k=0.688$), showing that both tests gave congruent results. It is worthy
205 mentioning that all samples that gave negative results by IPMA were also negative by
206 ELISA test; 6 out of 94 samples gave positive results by IPMA (low titres 1:20-1:60)
207 and negative by ELISA.

208

209 *2.4. DNA extraction and PCV2 quantitative PCR (qPCR)*

210 DNA from Spanish samples was extracted from 200 μ l of serum or 300 μ l of nasal PBS
211 solution (Nucleospin® Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) or
212 from 300 μ l of rectal PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH,
213 Germany), according to the manufacturers instructions. DNA from serum and nasal
214 swab samples was eluted in 100 μ l of elution buffer, and in 200 μ l for rectal swab
215 samples. DNA from Danish samples was extracted from 200 μ l of serum or 200 μ l nasal
216 PBS solution (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany) or 200 μ l of rectal
217 PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), according to
218 the instructions of the manufacturer. DNA was eluted with 200 μ l elution buffer.

219

220 PCV2 DNA was quantified using two previously described real time qPCR techniques
221 on Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2008) samples,
222 respectively. The performance of both qPCRs was compared in a previous study based
223 on field samples (Hjulsager et al., 2008). This latter work showed that the two qPCRs
224 gave comparable results showing a highly significant linear association between results

225 of the two assays. However, the method performed by the Danish laboratory had higher
226 sensitivity and yielded systematically higher PCV2 load values than the one used in the
227 Spanish laboratory (corresponding to a mean difference of $1.4 \log_{10}$ copies of PCV2 per
228 ml of sample).

229

230 *2.5. Statistical analyses*

231 Statsdirect (version 2.6.6, CamCode, Ashwell, UK) and SPSS 15.0 (SPSS Inc. Chicago,
232 USA) were used for statistical analyses. Analysis of variance with Bonferroni multiple
233 comparisons (for normally distributed variables) and Kruskal-Wallis and Mann-
234 Whitney tests (for non-normally distributed variables) were used to compare IPMA
235 titres, ELISA titres, lesional scores, antigen or DNA staining levels in lymphoid tissues,
236 and viral loads in sera, nasal swabs and rectal swabs between the three categories of
237 pigs studied (PMWS cases, wasted non-PMWS cases and healthy) in both countries.
238 Chi-square test was used to compare the proportions of positive qPCR results between
239 the three categories of pigs studied; when statistically significant different proportions
240 were detected, the Fischer's exact test was applied to compare in pairs the different
241 categories of pigs. The Fischer's exact test was also applied to compare the proportion
242 of viremic and non-viremic sows that had at least one PMWS case in their litter. Linear
243 associations between viral loads detected in the different samples, between lesions
244 characteristic of PMWS and amount of PCV2 in lymphoid tissues, and between total
245 antibody titres detected in sows and their piglets at 1 week of age, were determined by
246 Pearson's (normally distributed variables) and Spearman's (non-normally distributed
247 variables) correlations. To determine the diagnostic performance of serology and qPCR
248 techniques on clinically affected animals, a Receiver Operating Characteristic (ROC)
249 analysis was carried out using Statsdirect. For this, only wasted pigs were considered,

250 which perfectly fits a practical situation where a diagnosis of clinically suspected
251 animals at field is required. Thus, PMWS cases were considered as diseased, whereas
252 wasted non-PMWS cases were considered as non-diseased animals. The probability of
253 making a correct diagnosis was assessed through calculation of positive predictive value
254 (PV+) and negative predictive value (PV-). The level of significance for all analyses (α)
255 was set to $p < 0.05$.

256

257 **3. Results**

258 *3.1. Studied animals and histopathology*

259 A total of 108 and 118 animals were necropsied and pathologically characterised in
260 Spain and Denmark, respectively (Table 2). All Spanish PMWS outbreaks occurred at
261 the beginning of the fattening phase, whereas all Danish clinical outbreaks were
262 observed at nurseries. Mean \pm SD of weeks of age at necropsy were 10.6 ± 1.8 (PMWS:
263 10.6 ± 1.7 , healthy: 10.8 ± 2.0 , wasted non-PMWS: 10.3 ± 1.9) and 14.7 ± 2.5 (PMWS:
264 14.0 ± 2.1 , healthy: 14.7 ± 2.5 , wasted non-PMWS: 15.2 ± 2.7) in Denmark and Spain,
265 respectively. Accordingly, highest mortality ratios were observed at nurseries
266 (Denmark) and fattening facilities (Spain).

267

268 After histopathological examination, 35 out of 78 (45%) and 51 out of 91 (56%) pigs
269 showing wasting were diagnosed as PMWS cases in Spain and Denmark, respectively.
270 PMWS cases had higher amount of PCV2 in tissue (antigen or DNA staining), as well
271 as higher histopathological scores for lymphoid depletion and granulomatous infiltration
272 compared to wasted non-PMWS cases and healthy pigs from both countries ($p < 0.0001$).
273 Moreover, wasted non-PMWS pigs had higher global histopathological average scores
274 ($p < 0.0001$), as well as higher lymphoid depletion ($p < 0.02$) and granulomatous

275 infiltration ($p<0.01$) scores, than healthy pigs from both countries. The PCV2 amount in
276 lesions of wasted non-PMWS pigs was higher than in healthy pigs from Spain ($p<0.01$)
277 but not from Denmark ($p=0.5$). Surprisingly, 1 (Spain) and 2 (Denmark) pigs with good
278 clinical condition (apparently healthy) had a global average score >4 , showing both the
279 lesional score and the score for PCV2 amount, each above 2. These three animals were
280 finally not classified into the category of healthy pigs and were consequently excluded
281 from the statistical analyses due to the low number of cases with this situation.

282

283 3.2 PCV2 qPCR

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

300

301 Means of PCV2 load by qPCR in positive samples (figure 1) showed significant
302 differences between PMWS cases and healthy pigs at necropsy in serum, nasal and
303 rectal swabs from both countries ($p<0.001$). Furthermore, it was possible to distinguish
304 PMWS cases from healthy pigs in Danish farms at the sampling prior at the necropsy on
305 sera ($p<0.01$) and nasal swabs ($p<0,05$), but not in Spanish samples, even though a
306 tendency was observed for serum samples ($p=0.08$).

307

308 Positive and significant correlations were found in both countries among PCV2 viral
309 loads in sera and nasal swabs (Spain: $R=0.693$, $p<0.0001$; Denmark: $R=0.663$;
310 $p<0.001$), in sera and rectal swabs (Spain: $R=0.608$, $p<0.0001$; Denmark: $R=0.720$,
311 $p<0.0001$) and in nasal and rectal swabs (Spain: $R=0.593$; $p<0.0001$; Denmark:
312 $R=0.736$, $p<0.0001$) considering longitudinally collected samples. Moreover, positive
313 and significant correlations were also found between PCV2 amount detected in tissue
314 by ISH/IHC and viral loads detected by qPCR in sera (Spain: $R=0.625$, $p<0.0001$;
315 Denmark: $R=0.827$, $p<0.0001$), in nasal swabs (Spain: $R=0.573$, $p<0.0001$; Denmark:
316 $R=0.779$, $p<0.0001$) and in rectal swabs (Spain: $R=0.289$, $p<0.01$; Denmark: 0.507 ,
317 $p<0.0001$) collected at the moment of necropsy from both countries.

318

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

325

326 Positive PCV2 qPCR results were found in 2 out of the 87 (2.3%) and in 20 out of 68
327 (29.4%) studied sow's sera from Spain and Denmark, respectively (table 5). In
328 Denmark, 9 out of 20 sows with low but detectable viremia (45.0%) and 18 out of 48
329 sows with no detectable viremia (37.5%) had one or more PMWS affected pig in their
330 litter ($p=0.596$). Thus, there was no indication of any association between sow-viremia
331 and bearing of PMWS cases in the Danish study. Due to the low number of PCV2
332 positive sows in the Spanish data-set, a similar analysis based on Spanish data was not
333 possible.

334

335 3.3. PCV2 Serology

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

346

347 For the Spanish study, a significant and positive correlation was found between sow
348 PCV2 antibody titres and PCV2 antibody titres of their piglets at 1 week of age

349 (R=0.66, $p < 0.0001$). No correlation was found for antibody titers between Danish sow
350 and piglets (R=0.2, $p = 0.2$).

351

352 *3.5. Diagnostic performance of qPCR and serological tests*

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

360

361 None of the tests on the studied samples were specific and sensitive enough to diagnose
362 PMWS at the level of individual pigs in any of the two countries. Consequently, none of
363 the tests on the studied samples gave PV+ or PV- values higher than 90% in both
364 countries. On the other hand, taking into account the previously described bias between
365 the two qPCR assays of approximately 1.4 log copies PCV2/ml (Hjulsager et al., [2008](#))
366 qPCR on sera gave similar optimal cut-off for both qPCRs (6.21 in Spain and 7.43 in
367 Denmark). This fact led us to further explore the diagnostic performance of a
368 combination of qPCR in sera and serology tests for the Danish and the Spanish data-
369 sets. Results showed that the serial use of qPCR from sera and serology gave adequate
370 results in terms of specificity in both countries, but with an evident lack of sensitivity.
371 Thus, when Spanish qPCR serum load ≥ 6.21 and IPMA titres $\leq 1:1620$ were
372 simultaneously considered to confirm or discard a PMWS case, specificity and PV+
373 were 100%, but sensitivity was 42.9% and PV- was 68.3%. Regarding Danish tests,

374 when qPCR values in sera were ≥ 7.43 and ELISA titres were = 0, specificity was
375 =93.3%, PV+=83.3%, sensitivity=21.7% and PV-=43.8% .

376

377 **4. Discussion**

378 The lack of an effective and consistent experimental model to reproduce PMWS makes
379 it especially relevant to carry out epidemiological studies within PMWS affected farms.

380 Up to date, few longitudinal epidemiological data are available on PCV2 infection
381 dynamics in PMWS affected farms (Rodriguez-Arriola et al., 2002; McIntosh et al.,
382 2006; Carasova et al., 2007). In the present work, serology and qPCR techniques were
383 used to describe the evolution of PCV2 infection and excretion in histopathologically
384 well-characterized pigs (PMWS cases, wasted non-PMWS cases and healthy pigs) from
385 two different countries: Spain and Denmark. This is the first collaborative multi-centre
386 epidemiological study on PMWS, which has been carried-out on different farms and in
387 two different countries.

388

389 Overall, similar PCV2 dynamic patterns were observed between countries. Serological
390 analyses showed maternal antibody titres in all piglets at 1 week of age, which
391 decreased gradually and reached a minimum at approximately 6-7 weeks of age. PCV2
392 viral load in serum increased concurrently to waning of maternal antibody levels,
393 reaching the highest level at PMWS outbreak in all the three studied types of samples
394 (serum, and nasal and rectal swabs) in both countries. As expected (Brunborg et al.,
395 2004; Olvera et al., 2004), PCV2 load was higher in PMWS affected than in healthy
396 pigs at the moment of necropsy. Interestingly, even at the sampling previous to the
397 clinical outcome (9 and 11 weeks of age for Denmark and Spain, respectively), PMWS
398 cases had significant higher PCV2 load (Denmark) or PCV2 prevalence (Spain) than

399 healthy pigs, suggesting that PMWS affected pigs suffered from a higher viral load
400 and/or longer viremia than healthy ones.

401

402 A previous comparison of both qPCR showed that despite the results obtained by both
403 techniques had a good correlation, the Danish assay was more sensitive and yielded
404 systematically higher PCV2 viral loads than the Spanish assay (Hjulsager et al., 2008).

405 However, these differences in qPCR performance can not explain all the variation
406 between the findings in Spain and Denmark. Additional variation in results potentially
407 could be due to the employment of different extraction methods, which could also affect
408 the sensitivity of PCV2 detection (Fahle and Fischer, 2000; Cler et al., 2006).

409

410 PMWS was described as enzootic and epizootic presentations during the studied period
411 in Spain and Denmark, respectively (Segalés et al., 2007). Interestingly, the high
412 percentage of nasal Danish swabs positive by qPCR at younger ages agrees with a
413 previous work performed during the years of maximum PMWS affectation (epizootic
414 presentation) in Spain, which was based on conventional non-quantitative PCR on nasal
415 swabs (Sibila et al., 2004). Furthermore, present results showed a substantial delay in
416 PMWS age-presentation in Spain compared to Denmark. These data suggested the
417 existence of a relation between early infection and age at disease presentation. The
418 apparent higher PCV2 prevalence observed in young Danish piglets and sows suggested
419 that an earlier and higher PCV2 infection pressure was present in Danish farms
420 compared to Spanish ones, which might be the reason for an earlier PMWS presentation
421 and reflects the epidemiological disease presentation in both countries during the study
422 period.

423

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

438

439 The results of the present study indicated that the major spread of PCV2 occurred
440 between 4 and 6 weeks of age in Danish farms and between 7 and 11 weeks of age in
441 Spanish farms, which corresponded to the nursery phase (Denmark) and/or at the
442 beginning of fattening (Spain). However, the fact that some sows were viremic during
443 the lactating period together with PCV2 detection in piglets demonstrated that PCV2
444 infection was already present at pre-weaning ages and support the possible transmission
445 of virus from sows to nursing piglets (Larochelle et al., 2003; Sibila et al., 2004) and
446 also points out a potential vertical transmission (Pensaert et al., 2004; Shibata et al.,
447 2006). In fact, PCV2 DNA was repeatedly found in sera of pigs from 7 to 70 days of
448 life, further supporting the idea that some animals can be persistently infected under

449 field conditions, even in the presence of high levels of PCV2 specific antibodies
450 (Rodriguez-Arrijo et al., 2002; McIntosh et al., 2006). Those viremic pigs were
451 demonstrated to intermittently excrete PCV2 in nasal excretions and faeces, which
452 could potentially cause infection to their littermates and contribute to virus
453 dissemination in weaning and/or fattening facilities. On the other hand, no difference
454 between PMWS occurrence in piglets from viremic and non-viremic Danish sows was
455 found, which is in contradiction to a previously published field study (Calsamiglia et al.,
456 2007), probably due to cross-fostering practices which were allowed in Danish farms, in
457 contrast to the above-mentioned study.

458

459 PMWS cases had lower maternal antibody titres than healthy ones in both studied
460 countries, supporting previous studies indicating that total antibody titres may confer a
461 certain protection against PCV2 infection and clinical disease (Rodriguez-Arrijo et al.,
462 2002; Lopez-Soria et al., 2005; Calsamiglia et al., 2007). Maternal antibodies at the first
463 week of age were correlated with sow antibody levels in the Spanish study, but not in
464 the Danish one, probably as a consequence of cross-fostering practice in the Danish
465 study. Antibody titres after infection in Spanish pigs showed that non-PMWS affected
466 animals (both healthy and wasted non-PMWS affected pigs) had a higher antibody
467 response, being able to produce higher antibody titres than PMWS affected animals.
468 The fact that Spanish wasted non-PMWS and healthy pigs were as a mean 2 and 1
469 week, respectively, older than PMWS could contribute in explaining these differences at
470 the moment of necropsy, but not at 11 weeks of age. It is possible that wasted non-
471 PMWS affected animals, although infected with higher PCV2 loads than healthy pigs,
472 were able to overcome or limit the disease expression by the development of PCV2
473 antibodies, among other potential factors. However, these differences were not observed

474 in Danish farms. Taken together these findings suggest that total antibodies are not the
475 only factor involved in avoiding PMWS development (Meerts et al., 2006; Fort et al.,
476 2007). Other factors such as levels of neutralizing antibodies or cellular immunity,
477 although they were not evaluated in the present work, were previously demonstrated to
478 play an important role in PMWS development (Segalés et al., 2004; Fort et al., 2007).

479

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

497

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

519

520 Diagnostic performance of both qPCR and serology tests used in Denmark and Spain
521 was assessed to evaluate if they can be useful to diagnose PMWS in live animals. This
522 was especially relevant in the present work because most of samples were obtained

523 from live animals, fitting perfectly a practical situation in which veterinarians may be
524 able to diagnose PMWS without having to euthanize suspected animals. Taking into
525 account the bias among the qPCR methods used (Hjulsager et al., 2008), sera was the
526 only type of sample that gave an optimal cut-off value similar in both countries. Those
527 values were close to the threshold of 10^7 PCV2 copies per ml of sera previously
528 proposed (Brunborg et al., 2004; Olvera et al., 2004; Fort et al., 2007). Differences in
529 sampling procedure and material used could explained the different optimal cut-offs
530 values obtained by nasal and rectal swabs in both countries. Overall, our results
531 indicated that PCV2 qPCR (mainly from sera and nasal swabs) can give valuable
532 information related to PMWS status in a group of pigs, being able to differentiate a
533 population of PMWS affected from one of non-PMWS affected pigs during a PMWS
534 outbreak and even some days before. However, a desirable probability of making a
535 correct individual diagnosis higher than 90% was far from being achieved. The
536 combination of the qPCR technique and a serology test improved specificity and PV+,
537 indicating that such combination could be used to potentially confirm PMWS in live
538 PMWS-suspected animals, but with a high percentage of false negatives due to their
539 low combined sensitivity and PV-. Therefore, the lack of sensitivity and/or specificity
540 values observed from both tests used separately or combined suggested that qPCR
541 and/or serology tests are not able to substitute histopathology plus detection of PCV2 in
542 tissues for the individual PMWS diagnosis within PMWS affected farms.

543

544 The present report is the first descriptive infection dynamics study performed in
545 multiple PMWS affected farms from different countries. This work describes the
546 evolution of PCV2 infection, excretion and PCV2 antibodies in histopathologically
547 well-characterized pigs. Vaccines against PCV2 have been recently developed and are

548 under commercial use in different parts of the world. Understanding PCV2 infection
549 dynamics in the field is considered crucial for a proper design of vaccine strategies and
550 its corresponding monitoring.

551

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561

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704
705

Figure 1. Mean of positive Log₁₀(copies PCV2 per ml serum or per ml 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. **a,b,c** indicate statistical significant differences between groups ($p < 0.05$).

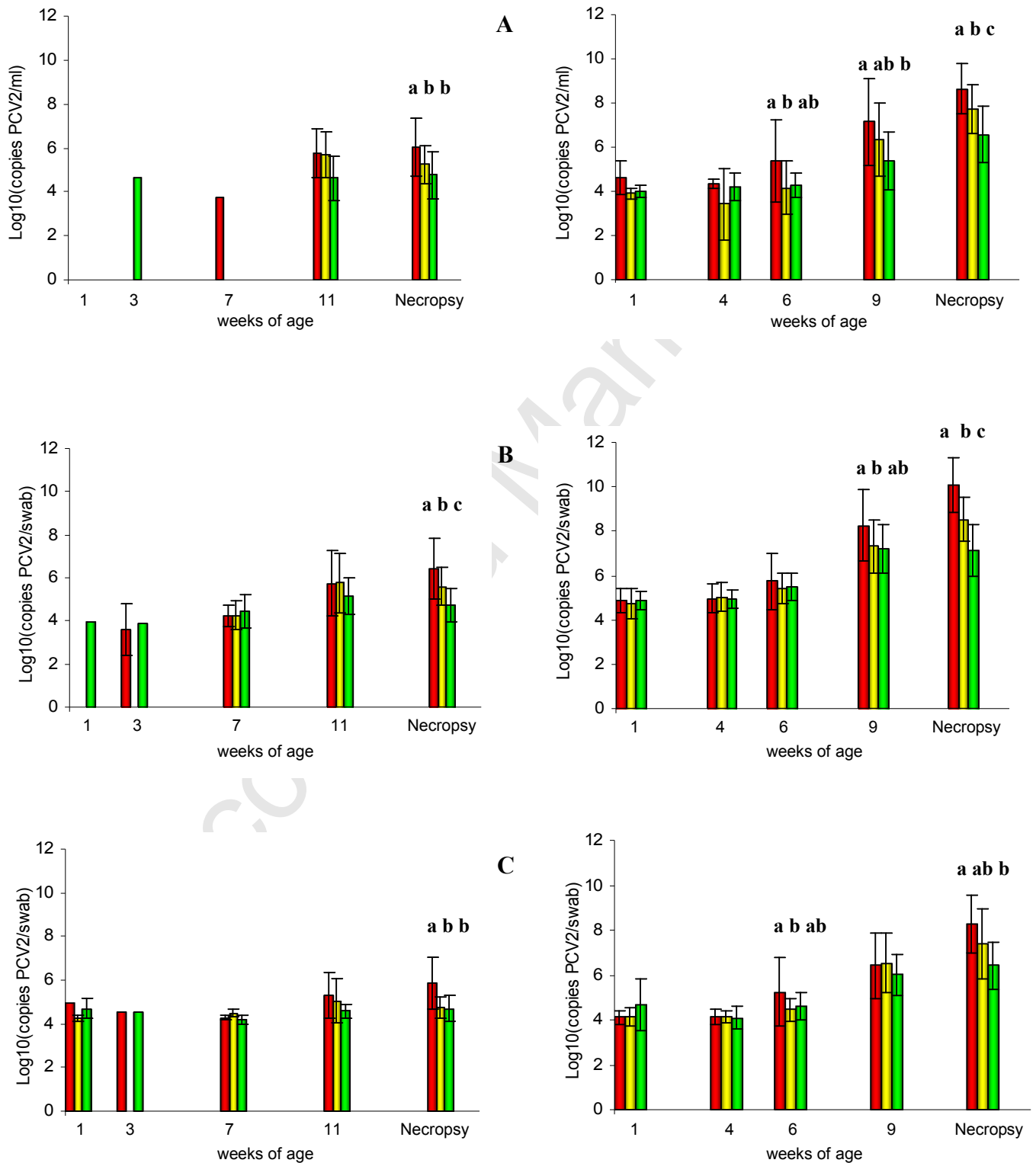


Figure 2. Mean \pm SD coded titres for porcine circovirus type 2 (PCV2) total antibodies in PMWS (—▲—), Wasted non-PMWS (—■—) and Healthy (---○---) pigs at the different samplings from Spanish (above) and Danish (below) farms. ^{a,b,c} Different letters in each sampling indicate significant differences between groups ($p < 0.05$).

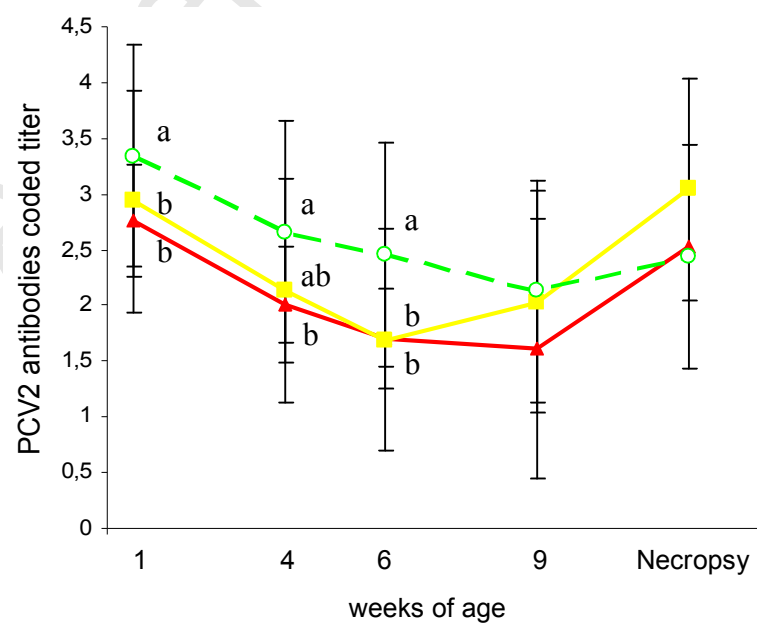
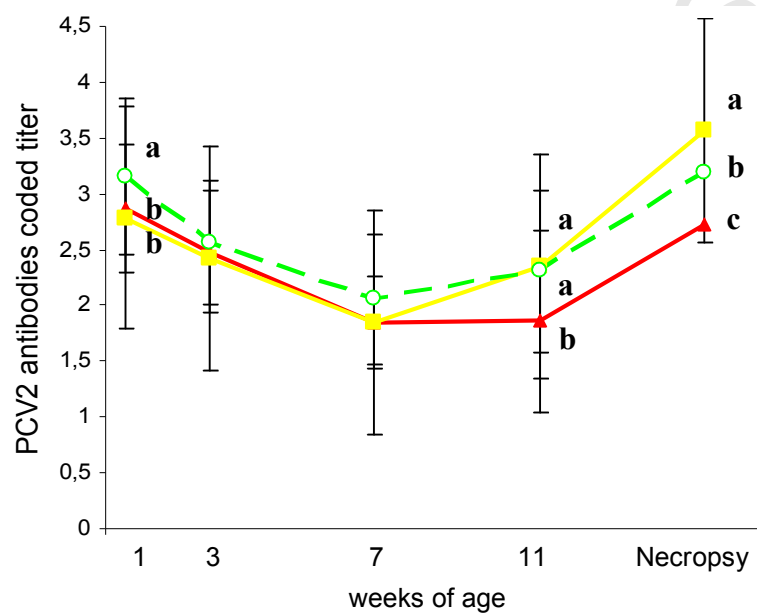


Table 1. Experimental design of the study in Denmark and Spain.

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

* In 2 out of 6 Spanish studied batches only males were followed up. Consequently, up to 21 sows were needed to select 100 pigs in those two batches.

** This range comprised 7 out of 8 studied Danish batches, but in one batch this number was 38.

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

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 intracellulari* (law), *Mycoplasma hyopneumoniae* (Myc), *salmonella spp.* (salm), Toxigenic *Pasteurella multocida* (PMT).

Farm	Health status	Production system	Number of sows	Batch	Weeks of age at necropsy	Number of necropsied pigs during PMWS outbreak			% Mortality**	
						PMWS	Wasted non-PMWS	Healthy	Nurseries	Fattening
SP-1	PRRSV+, ADV+, Myc+, PPV+, SIV+, Salm+	3 sites	2400	SP-1	18-21	3	8	5	1,9	8,0
SP-2	PRRSV+, ADV-, Myc+, PPV+, SIV+, Salm+	2 sites*	600	SP-2a	15-17	6	5	5	4,8	10,0
				SP-2b	13-15	3	7	5	2,9	17,4
				SP-2c	11-15	6	5	5	7,7	17,4
SP-3	PRRSV-, ADV-, Myc-, PPV+, SIV+, Salm+	2 sites	950	SP-3a	12-15	7	9	5	6,0	16,8
				SP-3b	12-15	10	9	5	1,9	16,8
Total number of studied animals in Spanish study						35	43	30		
DK-1	PRRSV+, Myc+, PPV+, SIV+, law+, Ap12+	1 site	570	DK-1	9-10	2	2	2	11,0	5,0
DK-2	PRRSV+, Myc+, PPV+, SIV+, law+, Ap6+	2 sites	700	DK-2	10-13	5	3	3	6,0	*** _
DK-3	PRRSV+, Myc+, PMT+, PPV+, SIV+, law+, Ap6+	3 sites	240	DK-3	10-13	18	20	5	22,0	4,5
DK-4	PRRSV+, Myc+, PPV+, SIV+, law+, Ap2+, Ap6+, PMT+	1 site	300	DK-4	10-13	9	3	5	15,0	7,0
DK-5	PRRSV+, Myc+, PPV+, SIV+, law+, Ap6+, Ap12+	2 site	570	DK-5	10-11	2	2	2	7,2	*** _
DK-6	PRRSV+, Myc+, PMT+, PPV+, SIV+, law+, Ap6+	2 sites	900	DK-6	10-13	7	8	4	5,4	*** _
DK-7	PRRSV+, Myc+, Ap2+, Ap12+, PPV+, SIV+, law+	1 site	400	DK-7	8-10	3	1	3	12,0	5,0
DK-8	PRRSV-, Myc-, PPV+, SIV+, law+	1 site	550	DK-8	9	5	1	3	6,0	*** _
Total number of studied animals in Danish study						51	40	27		

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

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

*** Not recorded data

Table 3. Histopathological scoring system used to evaluate the presence of PMWS-like lesions in studied lymphoid tissues of each necropsied pig.

Evaluated parameter	Scoring value				
	0	1	2	3	4
Lymphocyte depletion	Absence	lack of lymphoid follicles in <25% of the tissue section	lack of lymphoid follicles in 25 to 75% of the tissue section	lack of lymphoid follicles in >75% of the tissue	lack of lymphoid follicles
Granulomatous infiltration	Absence	mild and focal or multifocal granulomatous infiltration in <25 of the lymphoid follicles	multifocal granulomatous infiltration in 25 to 75% of the lymphoid follicles	granulomatous infiltration in >75% of the follicles	sections with diffuse granulomatous infiltration
ISH/IHC labelling	No labelling	low and focal or multifocal labelling in <25% of the follicles	labelling located exclusively at follicles (from 25 to 100% of the follicles) or labelling between 25 and 50 % tissue section	labelling at follicles and parafollicular areas in >50% tissue section	Diffuse labelling

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 are given in parentheses (PMWS, wasted non-PMWS and healthy pig, respectively) and differences indicated by superscripts.

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

*5th sampling correspond to necropsy moment in both countries

Table 5. Number of qPCR PCV2 positive sows in sera, mean of viral load of positive sows, and number of PMWS cases from viremic and non-viremic sows per studied batch.

Country	Batch	Number of PCV2 qPCR positive sows /Number studied sows	Viral load of positive sows (Log ₁₀ copies PCV2/ml) (mean±standard deviation)	Number of PMWS cases	
				From PCV2 positive sows	From PCV2 negative sows
Spain	sp-1a	0/12	-	0	3
	sp-2a	1/12	5.35	0	6
	sp-2b	0/12	-	0	3
	sp-2c	0/12	-	0	6
	sp-3a	0/20	-	0	7
	sp-3b	1/19	5.13	3	7
	Total	2/87	5.24±0.16	3	32
Denmark	dk-1a	2/8	4.16±0.6	2	0
	dk-2a	1/10	3.84	0	5
	dk-3a	0/10	-	0	18
	dk-4a	6/10	3.79±0.31	9	0
	dk-5a	6/10	3.87±0.12	1	1
	dk-6a	2/10	3.97±0.34	2	5
	dk-7a	1/4	3.79	1	2
	dk-8a	2/6	3.71±0.19	2	2
	Total	20/68	3.86±0.27	17	33

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 log₁₀ copies of PCV2 per ml sample.

Country	Sample	Test	Optimal cut-off	Sensitivity (CI _{95%})	Specificity (CI _{95%})	PV+ (CI _{95%})	PV- (CI _{95%})
Spain	Nasal swab	qPCR	≥5.9	68.6 (31.4-66.0)	74.4 (77.8-97.4)	68.6 (52.5-86.9)	74.4 (67.5-90.4)
	Rectal swab	qPCR	≥5.9	41.2 (24.7-59.3)	97.7 (87.7-99.9)	93.3 (68.1-99.8)	67.7 (55.3-80.2)
	Sera	qPCR	≥6.21	48.6 (31.4-66.0)	90.7 (77.8-97.4)	81.0 (58.1-94.6)	68.4 (55.5-81.4)
		IPMA	≤1:1620	73.5 (55.6-87.1)	88.4 (74.9-96.1)	83.3 (65.3-94.4)	80.9 (68.5-93.2)
Denmark	Nasal swab	qPCR	≥9.2	86.2 (68.3-96.1)	81.3 (54.4-96.0)	89.3 (71.8-97.7)	76.4 (50.1-93.2)
	Rectal swab	qPCR	≥8.1	50.5 (31.3-68.7)	82.4 (56.6-96.2)	83.3 (58.6-96.4)	48.3 (28.4-68.2)
	Sera	qPCR	≥7.43	91.3 (72.0-98.9)	46.7 (21.3-73.4)	72.4 (54.4-90.4)	77.8 (40.0-97.2)
		ELISA	=0	92.9(76.5-99.1)	27.8 (9.7-53.5)	66.7 (50.6-82.7)	71.4 (29.0-96.3)