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1 **A META-ANALYSIS ON EXPERIMENTAL INFECTIONS WITH PORCINE**
2 **CIRCOVIRUS TYPE 2 (PCV2)**

3
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14
15 **Short title:** A meta-analysis on PCV2 experimental infections
16

17 **Abstract**

18 A meta-analysis was performed with the aim to identify factors with a relevant
19 influence on the expression of clinical postweaning multisystemic wasting syndrome
20 (PMWS) under experimental conditions. Data from 44 studies were included in the analysis.
21 Several variables were studied: number of pigs in the experiment, intake of colostrum,
22 serological status against porcine circovirus type 2 (PCV2), strain of PCV2 used for
23 inoculation, the route and dose of inoculation, use of potential triggering factors (such as co-
24 infections, vaccinations, or immunomodulator products). Multiple correspondence analysis
25 and log-linear regression methods were used to establish the relationships between the studied
26 variables and the number of PCV2 infected pigs that developed PMWS. Based on the results
27 of the meta-analysis, the most successful animal experiment aimed to develop PMWS should
28 include: 1) colostrum-deprived pigs, 2) age of inoculation below three weeks, 3) high doses of
29 PCV2 inoculum, 4) PCV2 strain from genotype 1, and 5) co-infection with another swine
30 pathogen as a triggering factor.

31

32 **Keywords:** experimental infections / meta-analysis / porcine circovirus type 2 / postweaning
33 multisystemic wasting syndrome

34

35 1. Introduction

36 Porcine circovirus type 2 (PCV2) has been recognized as the essential infectious agent
37 of postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1999; Bolin et al.,
38 2001). This syndrome is a multifactorial disease that mainly affects nursery and fattening pigs
39 of almost all types of farms (Madec et al., 2000; Rose et al., 2003; López-Soria et al., 2005).
40 PMWS characteristic clinical signs are severe weight loss (wasting), pallor of skin, respiratory
41 distress, diarrhoea and, occasionally, jaundice (Harding et al., 1997). The morbidity and
42 mortality rates due to PMWS are highly variable from farm to farm, ranging from 4 to 30%,
43 depending on the type of farm, husbandry and management practices, co-infections with other
44 swine pathogens, or host genetics (Segalés et al., 2005).

45 Although PCV2 infection has been shown to be necessary for the full expression of
46 PMWS, it is known that the virus is present in both healthy and diseased pigs and herds (Rose
47 et al., 2003, Sibila et al., 2004). However, it has been traditionally difficult to reproduce the
48 severe clinical expression of the disease observed under field conditions through experimental
49 infections with PCV2 alone (Allan et al., 1999, Albina et al., 2001, Resendes et al., 2004).
50 Therefore, it has been suggested that other infectious or non-infectious factors must be
51 concomitantly influencing the development of PMWS in the field.

52 Experimental models are essential for researchers to study the pathogenesis of
53 infectious diseases, host immunological responses, as well as to evaluate the efficacy of
54 vaccines against an etiological agent. Up to date, however, it has been difficult to establish a
55 universal effective model for the reproduction of PMWS in swine. Few studies have been able
56 to reproduce PMWS by inoculating PCV2 alone (Bolin et al., 2001, Okuda et al., 2003, Lager
57 et al., 2007). The apparently most successful model, understanding it as the achievement of
58 clinical disease, implies the infection with PCV2 together with other swine pathogens, such as
59 porcine parvovirus (PPV) (Allan et al., 1999, 2003; Krakowka et al., 2000; Kim et al., 2003;

60 Opriessnig et al., 2004a; Hasslung et al., 2005), porcine reproductive and respiratory
61 syndrome virus (PRRSV) (Allan et al., 2000b; Harms et al., 2001; Rovira et al., 2002;
62 Opriessnig et al., 2006c) or *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004b), or the use
63 of non-infectious immunomodulators like the keyhole limpet hemocyanin in incomplete
64 Freund's adjuvant (KLH-ICFA) (Krakowka et al., 2001, 2002; Ladekjaer-Mikkelsen et al
65 2002; Grasland et al., 2005). However, a larger number of unsuccessful (lack of clinical
66 PMWS reproduction) experiments have been reported (Allan et al., 2000a,b; Loizel et al.,
67 2005; Ostanello et al., 2005; Opriessnig et al 2006a,c,d; Fernandes et al., 2007; Fort et al.,
68 2008).

69 Reasons for the inconsistent results achieved by independent studies are not yet
70 known, but there are several factors that may explain the different outcomes of experimental
71 infections with PCV2: origin of pigs, age of the animals at inoculation, immunological status,
72 genetic predisposition, PCV2 strain, type of inoculum, infectious dose, and route of
73 administration. In the present work we have performed a meta-analysis aimed at identifying
74 the most relevant factors that must be accounted for the successful reproduction of PMWS
75 under experimental conditions.

76

77 **2. Materials and methods**

78

79 **2.1. Data source**

80 The studies included in the present meta-analysis were obtained from peer reviewed
81 papers available at the public database PubMed (<http://www.ncbi.nlm.nih.gov/sites/entrez>) as
82 well as from not yet published results presented in scientific international congresses and
83 conferences which provided a detailed description of the methodology used and results
84 obtained (LeCann et al., 1998; Stockhofe-Zurwieden et al., 2003; Krakowka et al., 2006). The

85 articles retrieved dealt with infections in non-adult pigs for experimental reproduction of
86 PMWS. Inoculations in boars and/or pregnant sows were excluded from the analysis since
87 they were beyond the scope of this study. Only information regarding PCV2-inoculated pigs
88 with or without concomitant treatments was considered. Therefore, control pigs which were
89 mock inoculated, immunostimulated or infected with other swine viruses alone, or contact-
90 exposed to PCV2 were not included in the analysis. An experimental unit was defined as a
91 group of pigs that had received an identical treatment within the same study. Several variables
92 were annotated from each experimental unit:

- 93 1) Research group: 12 categories (RG1 to RG12).
- 94 2) Sample size: number of inoculated pigs in each experimental unit (three categories
95 used for multiple correspondence analysis: $N \leq 6$, $7 < N < 10$, or $N \geq 11$).
- 96 3) Age of pigs at inoculation: 4 categories (1-7 days, 1-3 weeks, 4-5 weeks, or > 5
97 weeks).
- 98 4) Colostrum deprivation: 2 categories (CD_YES or CD_NO).
- 99 5) Seronegative for PCV2 before inoculation: 2 categories (SN_YES or SN_NO). This
100 category is different from the previous one, since PCV2 seronegativity was achieved
101 either by colostrum deprivation or by colostrum antibody waning.
- 102 6) Route of inoculation: 2 categories (intranasal or other routes; latter one included
103 intraperitoneal, intratracheal, intramuscular, intralymphoid, or intrahepatic
104 inoculation routes).
- 105 7) PCV2 strain: 22 different strains.
- 106 8) PCV2 genotype: 2 categories (genotypes 1 or 2, Grau-Roma et al., 2008).
- 107 9) Dose of inoculum: 4 categories ($< 10E4$, $10E4-10E5$, $10E5-10E6$, or $> 10E6$ TCID₅₀
108 or equivalent per pig).

109 10) Type of experiment: 4 categories (PCV2 alone (PCV2), PCV2 plus
110 immunostimulation and/or vaccination (ISV), PCV2 plus co-infection (CI), or PCV2
111 plus immunostimulation and/or vaccination plus coinfection (ISV-CI).

112 11) Success: a response variable was defined to classify experimental units on the basis
113 of the percentage of PMWS-affected pigs. This variable allowed defining 4
114 categories (0%, 0-25%, 25-50%, and >50% of PMWS-affected pigs).

115

116 2.2. Statistical analyses

117

118 2.2.1. Univariate analysis

119 Main descriptive statistics were calculated for quantitative (mean, median, standard
120 deviation, maximum, and minimum) and for qualitative (frequency tables) variables.

121

122 2.2.2. Bivariate analysis

123 Contingency tables were used to explore the relationships between each independent
124 variable and the percentage of pigs affected (4 categories of success).

125

126 2.2.3. Multivariate Analysis

127 Multiple Correspondence Analysis (MCA) techniques combined with classification
128 methods were used to analyse the pattern of relationships of the related variables. MCA is a
129 descriptive, exploratory technique designed to analyse multi-way contingency tables with
130 cases as rows and categories of variables as columns (Greenacre 2007). The main objective of
131 this technique is to summarize a group of categorical variables, whose associations are jointly
132 considered, thus reducing initial variability. This analysis shows a group of subjects with
133 similar profiles regarding the chosen characteristics to describe them (categorical variables).

134 Classification methods were used to complement the results of the MCA making the
135 interpretation of the results easier. The advantage of classification methods is that they
136 incorporate a new element (the classes) easier to interpret than factorial axes. The
137 classification algorithm used was the mixed classification method (Lebart et al., 2000), a
138 combination of the algorithm of aggregation around gravity centres and the hierarchical
139 classification method. Nominal active variables used for MCA and direct hierarchical
140 classification were: age at inoculation, colostrum deprivation, PCV2 serological status, route
141 of inoculation, PCV2 genotype, dose of PCV2 inoculum, and type of experiment. Other
142 variables were considered as illustrative variables which were not used in the analysis but
143 were helpful for interpretation of results. These illustrative variables were: research group,
144 sample size, PCV2 strain, severity of PMWS, and the percentage of affected pigs (variable
145 success). The SPAD software (*Système Portable pour l'Analyse des Données, Centre*
146 *International de Statistiques et d'Informatique Appliquées (C.I.S.I.A.), Saint Mandé, France*)
147 was used for classification and multiple correspondence analyses.

148

149 2.2.4. Poisson regression models

150 A Poisson regression model (Cameron and Trivedi, 1998) was established using the
151 count of affected pigs following PCV2 inoculation as a response variable. The presence of
152 over-dispersion of the response variable was taken into account in the model. The sample size
153 used in the analysed experiments was highly heterogeneous among them and was, therefore,
154 considered as an offset term of the model. Due to the fact that experimental units coming
155 from a certain experiment are correlated, dependencies between non-independent
156 observations were also considered in the model. Firstly, a Poisson regression model was
157 established separately for each covariate to identify those that had a significant effect on the
158 number of affected pigs following PCV2 inoculation. Secondly, a global Poisson regression

159 model was fitted including all covariates simultaneously. This strategy allowed us to account
160 for possible interactions between variables non-detectable under the bivariate model
161 approach. Then, after a selection procedure, only those variables that remained significant
162 were kept in the final global model.

163 The GLIMMIX procedure of the SAS software (SAS Institute Inc., Cary, NC, USA)
164 was used for fitting the Poisson regression models. The offset parameter was introduced into
165 the model statement and the random statement was used for taking into account the possible
166 non-independence among observations and the presence of over-dispersion. The significance
167 level was set to 0.05.

168

169 **3. Results**

170

171 3.1. Descriptive statistics

172 A total of 100 experimental units, derived from 44 independent studies, were included
173 in the analyses (Table 1). Sixty percent of the experimental units were performed by only two
174 Research groups (RG1 and RG2), which accounted for 31% and 29% of them, respectively.
175 The remaining 40% of experimental units were done by 10 different Research Institutes.

176 Sample size was highly variable between experimental units, ranging from 3 to 58
177 pigs, with a mean sample size of 10.02 inoculated pigs per experimental unit. The age of pigs
178 at inoculation day was also variable ranging from one day to more than five weeks old.

179 With regards to the source of the pigs used, animals were fed with colostrum in 61%
180 of the experimental units, among which 65.6% (40 out of 61) and 34.4% (21 out of 61) were
181 seronegative and seropositive against PCV2 at the time of challenge, respectively. As
182 expected, all colostrum-deprived pigs (in 39 out of 100 experimental units, 39%) were
183 seronegative against PCV2 at the moment of inoculation.

184 The effect of genetics could not be considered because in most studies the breed of the
185 pigs used was not specified.

186 As much as 22 different PCV2 strains have been tested, although the ISU-40895 and
187 the Stoon-1010 ones have been the most extensively used, comprising more than 50% of the
188 experimental units (28 and 27 experimental units, respectively). Given the high level of
189 imbalance on PCV2 strain data we considered the genotype of the virus (Olvera et al., 2007;
190 Grau-Roma et al., 2008). Two strains from 3 different works were not classified in any
191 phylogenetic group since their genomic sequence was not available (Balasch et al., 1999;
192 Rovira et al., 2002; Ostanello et al., 2005). Almost 80% of the remaining strains corresponded
193 to genotype 2.

194 The most frequent route of inoculation was the intra- or oro-nasal route (87%), which
195 used variable doses of inoculum ranging from 10^2 TCID₅₀/pig to 10^6 TCID₅₀/pig. In 57% of
196 the experimental units, PCV2 was inoculated alone; the remaining most frequent designs used
197 PCV2 and co-infection with PPV (12%), immunostimulation with KLH-ICFA (8%), co-
198 infection with PRRSV (7%), and co-infection with *M. hyopneumoniae* (6%).

199 Only 38% of the experimental infections resulted in reproduction of PMWS, with a
200 mean percentage of affected pigs of 15.1%.

201

202 3.2. Bivariate analysis

203 Table 2 summarizes the distribution of the number and percentage of experimental
204 units within each variable category classified by the percentage of PMWS-affected pigs (four
205 categories). More than 80% of the experimental units that reproduced overt signs of PMWS in
206 more than 50% of the PCV2-infected pigs, inoculated the virus at ages comprised between the
207 first day and 3 weeks of life (9 out of 11 experimental units). Conversely, none of the

208 experimental units inoculating pigs older than 5 weeks of age could reproduce disease in such
209 a high percentage of pigs.

210 More than 50% (21 out of 39) of experimental units using colostrum-deprived pigs
211 could reproduce PMWS in at least one pig. Of them, 23.1% yielded more than 50% of
212 affected pigs. Conversely, in colostrum-fed models, only 27.9% (17 out of 61) succeeded in
213 the reproduction of disease in at least one pig and only 3.3% of the cases with more than 50%
214 of affected pigs. Similarly, models using PCV2-seronegative pigs were able to reproduce
215 PMWS in approximately 45% of the cases, with 13% (10 out of 77) of them yielding more
216 than 50% of affected pigs, whereas inoculation of PCV2-seropositive pigs only led to 13% of
217 successful cases (4.3% with more than 50% of affected pigs).

218 With regards to the PCV2 genotype, a higher percentage of successful experiments
219 (50%, 10 out of 20) were annotated in experiments using a PCV2 inoculum from genotype 1
220 compared to those experiments using strains from genotype 2 (36%, 27 out of 75), despite the
221 lower number of experiments using PCV2 strains belonging to genotype 1. Although similar
222 percentages of affected pigs were achieved with inoculums from both genotypes, they were
223 slightly higher for those experiments using inoculums from genotype 1. While 30% (6 out of
224 20) of the experimental units using an inoculum from genotype 1 achieved more than 25% of
225 affected pigs, only 18.7% (14 out of 75) of experimental units using a PCV2 inoculum from
226 genotype 2 reached this percentage of affected pigs.

227 If dose of inoculum is taken into account, it can be observed that the higher the dose of
228 PCV2, the higher the percentage of PMWS-affected animals. However, several experiments
229 have been proven to be successful with low doses of PCV2 (Allan et al., 1999; Harms et al.,
230 2001).

231 Finally, some experiments inoculating PCV2 alone reproduced PMWS (26.3% of total
232 PCV2-alone experimental units), but the vast majority of successful experiments were those

233 in which co-infection or immunostimulation-vaccination models were used (75% and 41.7%
234 of successful experiments recorded within each model, respectively). Interestingly, co-
235 infections produced higher percentages of PMWS-affected pigs (31.2% of experimental units
236 with more than 50% of affected pigs) than the use of immunomodulators or
237 vaccines/adjuvants (16.7%).

238

239 3.3. Multivariate Analysis

240 Figure 1 shows the result of the MCA in a two dimensional plane, according to the
241 two first axes of the MCA (32.9% of inertia, Table 3). It can be seen that the percentage of
242 PMWS affected pigs is positively correlated to the first axis (horizontal) and negatively
243 correlated to the second axis (vertical). The nominal active modalities that appear to be more
244 associated to the percentage of affected pigs are the ones that fall into the right and bottom
245 square of Figure 1 (i.e.: 1-7 days, CD_YES, SN_YES, 10E5-10E6 TCID50, >10E6 TCID50,
246 CI, and ISV). This figure, however, should be interpreted with caution due to the fact that
247 only information on the first two axes is used and a small proportion of variability of data is
248 explained by them. For classification and statistical description of classes the first seven axes
249 of the MCA analysis, which jointly explained 76.3% of total data variability, were used
250 (Table 3). Results of the mixed classification method are shown in Table 4. Experimental
251 units were assigned, based on the similarity of their experimental design (i.e., similar
252 modality characteristics), in four different classes (namely I, II, III, and IV). Two main
253 classes, II and IV, accounted for 30% and 42% of the total number of experimental units,
254 respectively. These two classification groups appeared to discriminate experimental units by
255 their level of success on reproducing PMWS.

256 Experimental units included in class II were characterized by the use of piglets fed
257 with colostrum, inoculated at an older age than 5 weeks and that received low to moderate

258 doses of PCV2 inoculum (10^4 - 10^5 TCID₅₀/pig). Seventy percent (21 out of 30) of the
259 experimental units associated to class II did not reproduce PMWS disease (all inoculated
260 piglets remained healthy throughout the experiment). Amongst the remaining 30% (9 out of
261 30) of experiments within this class, the average percentage of affected pigs was 2.5%. Thus,
262 class II mostly grouped non-successful experimental units.

263 Conversely, class IV was mainly associated to successful experiments. The most
264 representative modalities of this class were deprivation of colostrum, inoculation during the
265 first week of life, and inoculation of high doses of PCV2 ($>10^6$ TCID₅₀/pig). Remarkably,
266 more than 80% of all the reported experimental units with rates of success above 50% of
267 affected pigs were grouped in this class, representing 21.4% of all the experimental units
268 associated to class IV (9 out of 42). Finally, the average percentage of affected pigs of all
269 experimental units belonging to class IV was considered high (23.8%).

270 The route of inoculation and the PCV2 genotype did not appear to be relevant
271 variables in any class because identical modalities (genotype 2 and intranasal route of
272 inoculation) were present in both class II and class IV groups.

273 No remarkable association with the rate of success on reproducing PMWS was found
274 for the remaining classes I and III.

275

276 3.4. Poisson regression models

277 The response variable of the models (affected number of piglets) was assumed Poisson
278 distributed. However, the response variable was overdispersed with respect to the Poisson
279 distribution (deviance/df = 2.38). To model this situation we allowed the variance function to
280 have a multiplicative overdispersion factor Φ : $\text{Var}(y) = \Phi \mu$. This is the NB1 model described
281 by Cameron and Trivedi (1998). The standard errors of the parameters fitted by the Poisson
282 regression model were corrected by using the estimated value of Φ .

283 In the individual model, variables with a significant effect on the number of affected
284 pigs were, in decreasing order of significance: intake of colostrum ($F=13.7$, $P=0.0005$), type
285 of experiment ($F=4.5$, $P=0.007$), age at inoculation ($F=3.1$, $P=0.0357$), and serological status
286 of pigs before infection ($F=4.0$, $P=0.0495$). PCV2 genotype, route of inoculation, and dose of
287 inoculum used for infection of pigs did not have a significant individual effect. The estimated
288 differences of least squares means (β) and the ratio of the expected number of affected pigs
289 ($\exp(\beta)$) are displayed in Table 5. As it is shown, the ratio of expected number of PMWS
290 affected pigs was about four times higher ($\exp(\beta)=4.3$, $P=0.0005$) for colostrum-deprived pigs
291 compared to colostrum-fed pigs. Similarly, seronegative piglets were 3.5 times ($P=0.0495$)
292 more prone to develop PMWS after infection with PCV2 than seropositive piglets.
293 Furthermore, the mean number of affected pigs was significantly higher in piglets inoculated
294 within the first 3 weeks of age compared to those inoculated at older ages than 5 weeks. With
295 regards to the type of experiment, co-infections with other swine viruses significantly resulted
296 in higher expected mean number of affected pigs compared to experiments inoculating with
297 PCV2 alone ($\exp(\beta)=3.6$, $P=0.0006$). Co-infections tended to produce two times more
298 affected pigs than experiments using other immunostimulant factors ($\exp(\beta)=2.2$, $P=0.0791$).

299 In the global model, the only variables that remained significant were colostrum intake
300 status of pigs ($F=28.8$, $P<0.0001$) and genotype of PCV2 strain ($F=12.0$, $P=0.0008$). The
301 effect of colostrum deprivation was even more marked in the global analysis than in the
302 individual analysis, with a mean expected number of affected pigs almost six-fold higher in
303 colostrum-deprived pigs compared to colostrum-fed pigs ($\exp(\beta)=5.8$, $P<0.0001$, Table 5).
304 The PCV2 genotype effect, that was not significant in the individual analysis, now had a
305 significant effect when the intake of colostrum was jointly considered in the model. As shown
306 in Table 5 the ratio of expected number of affected pigs was more than three-fold higher for
307 PCV2 genotype 1 compared to genotype 2 ($\exp(\beta)=3.5$, $P=0.008$). The interaction effect

308 between colostrum deprivation and PCV2 genotype was included in the model but did not
309 reach the statistical threshold for significance (data not shown).

310

311 **4. Discussion**

312

313 Meta-analysis is a powerful statistical tool that has become widely used for the
314 validation of previous research on a common topic and has been applied to multiple areas of
315 knowledge including epidemiology (Greenland, 1987; Blettner et al., 1999). To our
316 knowledge, this is the first meta-analysis done to evaluate the performance of experimental
317 infections with an infectious agent in pigs. Two statistical approaches were used, including a
318 multiple correspondence analysis (MCA) to summarize the relationships between the studied
319 variables, and log-linear regression models to determine the effect of significant variables on
320 the number of PMWS-affected pigs. Both methodologies used are complementary. MCA
321 allows the characterization of groups of papers sharing similar characteristics and regression
322 techniques model the frequency of success. By their combination a better understanding of
323 data structure is achieved, thus facilitating the interpretation of results (Panagiotakos and
324 Pitsavos, 2004).

325 The MCA analysis indicated that the main common characteristics shared by
326 successful experiments (i.e., experiments that reproduce PMWS clinical signs in at least one
327 PCV2-inoculated animal) are the inoculation of colostrum-deprived pigs during the first week
328 of age with high doses of PCV2 inoculum. These results were mostly confirmed by regression
329 models. In this sense, the intake of colostrum and the age at inoculation had a significant
330 individual effect on the expected number of pigs that develop PMWS after infection with
331 PCV2. In addition, the type of experiment (co-infections with PCV2 and other viruses, or the
332 use of immunomodulators or vaccine/adjuvants) also had a significant individual effect.

333 Passively acquired antibodies through colostrum ingestion confer protection against
334 viral infections to newborn piglets (Ward et al., 1996; Parreno et al., 1999; Suradhat and
335 Damrongwatanapokin, 2003). The presence of antibodies to PCV2 has been shown to reduce
336 the peak viremia levels (McKeown et al., 2005) and to protect pigs from development of
337 severe PCV2-associated lesions (Allan et al., 2002; Opriessnig et al., 2006b). This situation
338 would explain why naturally PMWS-affected pigs are usually aged between 5 to 12 weeks
339 (Segalés et al., 2005), once maternal derived antibodies waned; however, a single case of
340 diseased pigs at earlier ages has been reported (Hirai et al., 2001). The higher potential of
341 success obtained for inoculations at younger ages could be due to the fact that colostrum-
342 deprived pigs are used to assure that no maternal immunity was acquired by piglets before
343 infection. In contrast, inoculations at older ages usually imply that piglets have been fed with
344 colostrum. This latter fact means that, in most cases, piglets have acquired maternal
345 antibodies against PCV2 and certain levels of maternal immunity (maybe not detectable by
346 current laboratorial techniques) that may remain at the time of inoculation at older ages. In
347 this sense, only one experiment has been reported in which caesarean-derived, colostrum-
348 deprived (CD-CD) piglets were inoculated at 8 weeks of age; however, none of the infected
349 piglets developed PMWS (Progranichnyy et al., 2000).

350 The dose of inoculum was not a significant variable in the regression models, although
351 it has been suggested that the severity of the disease is dose-dependent and, for most of the
352 cases, higher doses of inocula led to reproduction of PMWS experimentally (Albina et al.,
353 2001). However, the administration of relatively high amounts of inocula does not always
354 guarantee the development of PMWS (Resendes et al., 2004; Yu et al., 2007; Fernandes et al.,
355 2007). Conversely, Allan et al. (1999) could reproduce PMWS by administering only $10^{2.4}$
356 TCID₅₀ to each infected piglet.

357 The use of co-infections (Allan et al., 1999; 2003; Krakowka et al., 2000; Harms et al.,
358 2001; Rovira et al., 2001; Kim et al., 2003; Stockhofe-Zurwieden et al., 2003, Opriessnig et
359 al., 2004a; 2004b; Hasslung et al., 2005), immunomodulators (Krakowka et al., 2001, 2002;
360 Nielsen et al., 2003; Grasland et al., 2005; Loizel et al., 2005), or vaccine adjuvants
361 (Krakowka et al., 2006; 2007; Hoogland et al., 2006) have been proven effective to reproduce
362 PMWS in several experiments. However, many authors have reported that stimulation of the
363 immune system does not have a critical effect on intensifying the disease (Ladekjaer-
364 Mikkelsen et al., 2002; Resendes et al., 2004; Loizel et al., 2005; Fernandes et al., 2007). The
365 underlying mechanism by which activation of the host immune system interacts with PCV2
366 and empowers its replication still remains unsolved. However, results of the meta-analysis
367 study indicate that these schemes have a higher potential for reproducing PMWS than the
368 inoculation of PCV2 alone.

369 An interesting finding was that the genotype of the PCV2 strain used for inoculation
370 was significant when the intake of colostrum was jointly considered in the global analysis. In
371 contrast, PCV2 genotype did not have an individual significant effect, probably due to the low
372 number of experiments using PCV2 strains from genotype 1. Therefore, it is very likely that
373 no enough statistical power was available to detect such differences. However, different
374 PCV2 genotypes seem to differ in pathogenicity, as it has been suggested by both *in vitro*
375 (Meerts et al., 2005) and *in vivo* experiments (Hasslung et al., 2005; Opriessnig et al., 2006;
376 Lager et al., 2007). Therefore, further studies should be performed to elucidate if certain
377 PCV2 strains have a higher potential of developing PMWS under experimental conditions.

378 The main limitation of this meta-analysis stems from the inherent limitations of the
379 publications included in the analysis. Our conclusions could be biased due to the fact that
380 negative results obtained from experiments with identical experimental design to those with
381 positive results are finally not published. Another reason for bias in our study is the fact that

382 most of the experiments included in this meta-analysis were reported by few research groups,
383 which tend to use a determined strategy (successful or not) and their relative weight could
384 have had an important impact on the results.

385 Finally, several factors could not be studied because they were not explicitly
386 mentioned in a substantial number of publications (i.e., genetics of the pigs and number of
387 passages of the virus inoculum). Genetic susceptibility to suffer from PMWS has been
388 pointed out both under field (Madec et al., 2000; López-Soria et al., 2005; McIntosh et al.,
389 2006; Zhou et al., 2006) and experimental (Opriessnig et al., 2006b) conditions. On the other
390 hand, it has been demonstrated that, after serial cell passages, the original PCV2 virus
391 experiences adaptive genetic changes that can lead to virulence attenuation *in vivo* (Fenaux et
392 al., 2004). The number of PCV2 passages in cell culture for the corresponding inocula was
393 available in a very few number of publications.

394 In summary, this meta-analysis contributes to a better understanding of the factors that
395 have a major influence on the successful development of PMWS under experimental
396 conditions. Specifically, the highest likelihood to achieve PMWS in an experiment includes
397 pigs younger than 3 weeks of age, colostrum-deprived, inoculated with high doses ($>10^5$
398 TCID₅₀/pig) of PCV2 from genotype 1 and, preferably, in co-infection with another porcine
399 pathogen.

400

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635 **Tables**

636 Table 1. List of references included in the meta-analysis. The research group (RG) and the
 637 number of experimental units (N) of each study are specified.

Research Group	References	N	Research Group	References	N
RG1	Fenaux et al 2002	3	RG2	Krakowka et al 2002	4
RG1	Fenaux et al 2003	1	RG2	Krakowka et al 2005	5
RG1	Fenaux et al 2004	4	RG3	Balasz et al 1999	1
RG1	Lager et al 2007	2	RG3	Fernandes et al 2008	2
RG1	McKeown et al 2005	2	RG3	Fort et al 2008	4
RG1	Opriessnig et al 2004a	3	RG3	Resendes et al 2004	2
RG1	Opriessnig et al 2004b	2	RG3	Roca et al 2004	3
RG1	Opriessnig et al 2006a	2	RG3	Rovira et al 2001	2
RG1	Opriessnig et al 2006b	6	RG4	Albina et al 2001	2
RG1	Opriessnig et al 2006c	3	RG4	Grasland et al 2005	2
RG1	Opriessnig et al 2006d	2	RG4	Le Cann et al 1998	2
RG1	Yu et al 2007	1	RG4	Loizel et al 2005	2
RG2	Allan et al 1995	1	RG5	Bolin et al 2001	1
RG2	Allan et al 1999	2	RG5	Harms et al 2001	2
RG2	Allan et al 2000a	2	RG5	Progranichnyy et al 2000	1
RG2	Allan et al 2000b	2	RG6	Ladekjaer-Mikkelsen et al 2002	2
RG2	Allan et al 2003	2	RG7	Stockhofe-Zurwieden et al 2003	5
RG2	Allan et al 2007	2	RG8	Ostanello et al 2005	2
RG2	Ellis et al 1999	1	RG9	Magar et al 2000	1
RG2	Hasslung et al 2005	2	RG10	Wang et al 2007	2
RG2	Krakowka et al 2000	3	RG11	Okuda et al 2003	1
RG2	Krakowka et al 2001	3	RG12	Kim et al 2003	1

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648 Table 2. Distribution of the number of experimental units (and row percentage) within each
 649 variable category classified by the percentage of PMWS-affected pigs.

650 ^a PCV2: PCV2 alone, ISV: PCV2 + immunostimulator or vaccination, CI: co-infection, ISV-

651 CI: combination of categories ISV and CI.

Variables	Percentage of PMWS-affected pigs				Total (N=100)
	0%	< 25 %	25-50 %	> 50 %	
Age at inoculation					
1-7 days	16 (53.3)	6 (20.0)	2 (6.67)	6 (20.0)	30
1-3 wk	7 (46.7)	3 (20.0)	2 (13.3)	3 (20.0)	15
4-5 wk	15 (62.5)	4 (16.67)	3 (12.5)	2 (8.33)	24
> 5 wk	24 (77.4)	5 (16.1)	2 (6.5)	0 (0)	31
Colostrum deprivation					
NO	44 (72.1)	10 (16.4)	5 (8.2)	2 (3.3)	61
YES	18 (46.1)	8 (20.5)	4 (10.3)	9 (23.1)	39
Seronegative for PCV2					
NO	20 (87.0)	2 (8.7)	0 (0)	1 (4.3)	23
YES	42 (54.5)	16 (20.8)	9 (11.7)	10 (13.0)	77
Route of inoculation					
intranasal	53 (60.9)	15 (17.2)	8 (9.2)	11 (12.6)	87
other	9 (69.2)	3 (23.1)	1 (7.7)	0 (0)	13
Genotype					
1	10 (50.0)	4 (20.0)	4 (20.0)	2 (10.0)	20
2	48 (64.0)	13 (17.3)	5 (6.7)	9 (12.0)	75
Dose (TCID₅₀/pig)					
< 10E4	5 (41.7)	2 (16.7)	4 (33.3)	1 (8.3)	12
10E4 – 10E5	18 (66.7)	5 (18.5)	2 (7.4)	2 (7.4)	27
10E5 – 10E6	19 (65.5)	4 (13.8)	3 (10.3)	3 (10.3)	29
> 10E6	11 (57.8)	4 (21.1)	0 (0)	4 (21.1)	19
Type of experiment^a					
PCV2	42 (73.7)	8 (14.0)	5 (8.8)	2 (3.5)	57
ISV	14 (58.3)	6 (25.0)	0 (0)	4 (16.7)	24
CI	4 (25.0)	3 (18.8)	4 (25.0)	5 (31.2)	16
ISV-CI	2 (66.7)	1 (33.3)	0 (0)	0 (0)	3

652 Table 3. Eigenvalues, individual (% inertia) and cumulative (Cum. % inertia) proportion of
653 explained inertia of the first seven axis of the multiple correspondence analysis.

Axis	Eigenvalue	% inertia	Cum. % inertia
1	0.40	18.7	18.7
2	0.30	14.2	32.9
3	0.25	11.9	44.7
4	0.19	9.1	53.8
5	0.18	8.6	62.4
6	0.16	7.6	69.9
7	0.14	6.4	76.3

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673 Table 4. Results of the mixed classification method.

Number of experimental units within class	Percentages ^a		Number of cases within modality	Modality	Variables ^b
	Cla/Mod	Mod/Cla			
Class I: 14					
8	66.7	57.1	12	<10E4 TCID ₅₀	Dose of PCV2 inoculate
14	23.0	100.0	61	NO	Colostrum deprivation
9	37.5	64.30	24	4-5 wk	Age at inoculation
7	35.0	50.0	20	1	Genotype
Class II: 30					
26	83.9	86.7	31	>5 wk	Age at inoculation
22	81.5	73.3	27	10E4-10E5 TCID ₅₀	Dose of PCV2 inoculate
29	47.5	96.7	61	NO	Colostrum deprivation
28	37.3	93.3	75	2	Genotype
30	34.5	100.0	87	intranasal	Route of inoculation
25	40.3	83.3	62	0%	Success
15	50.0	50.0	30	>11	Sample size
	2.5 (6.9)	15.1 (25.8)			Percent (s.e.) of affected pigs
Class III: 14					
13	100.0	92.9	13	other routes	Route of inoculation
9	45.0	64.3	20	1	Genotype
8	33.3	57.1	24	4-5 wk	Age at inoculation
13	21.3	92.9	61	NO	Colostrum deprivation
Class IV: 42					
37	94.9	88.1	39	YES	Colostrum deprivation
29	96.7	69.1	30	1-7 days	Age at inoculation
					Dose of PCV2 inoculate
17	89.5	40.5	19	>10E6 TCID ₅₀	inoculate
40	53.3	95.2	75	2	Genotype
42	48.3	100.0	87	intranasal	Route of inoculation
9	81.8	21.4	11	>50%	Success
	23.8 (30.6)	15.1 (25.8)			Percent (s.e.) of affected pigs

674 ^a Cla/Mod: percentage of experiments classified in this class out of all the experiments
675 that display the characteristic, Mod/Cla: percentage of experiments that display a
676 determined characteristic within a class.

677 ^b s.e.: standard error

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682 Table 5. Differences of Least Squares Means between categories of variables significant in
 683 the individual and global models.

Variables	Estimate (β)	S.E.	t Value	P value	Exp(β)
Individual Modelisation					
Colostrum deprivation					
YES vs. NO	1.5	0.4	-3.7	0.0005	4.3
Seronegative for PCV2					
YES vs. NO	1.3	0.6	-2.0	0.0495	3.5
Age at inoculation					
1-3 wk vs. 1-7days	0.1	0.6	0.2	0.8163	1.1
1-3 wk vs. 4-5 wk	0.7	0.6	1.2	0.2460	2.1
1-3 wk vs. >5 wk	1.7	0.6	2.7	0.0094	5.5
1-7days vs. 4-5 wk	0.6	0.6	1.0	0.3156	1.8
1-7days vs. >5 wk	1.6	0.6	2.6	0.0113	4.8
4-5 wk vs. >5 wk	1.0	0.6	1.6	0.1268	2.7
Type of experiment^a					
CI vs. PCV2	1.3	0.4	-3.7	0.0006	3.6
ISV vs. PCV2	0.5	0.4	-1.2	0.2422	1.6
ISV-CI vs. PCV2	0.3	1.3	-0.2	0.8227	1.3
CI vs. ISV	0.8	0.5	1.8	0.0791	2.2
CI vs. ISV-CI	1.0	1.2	0.8	0.4257	2.7
ISV vs. ISV-CI	0.2	1.3	0.2	0.8751	1.2
Global modelisation					
Colostrum deprivation					
YES vs. NO	1.8	0.3	-5.4	<0.0001	5.8
Genotype					
1 vs. 2	1.2	0.4	3.5	0.0008	3.5

684 ^a PCV2: PCV2 alone, ISV: PCV2 plus immunostimulator or vaccination, CI:

685 PCV2 plus co-infection, ISV-CI: combination of categories ISV and CI.

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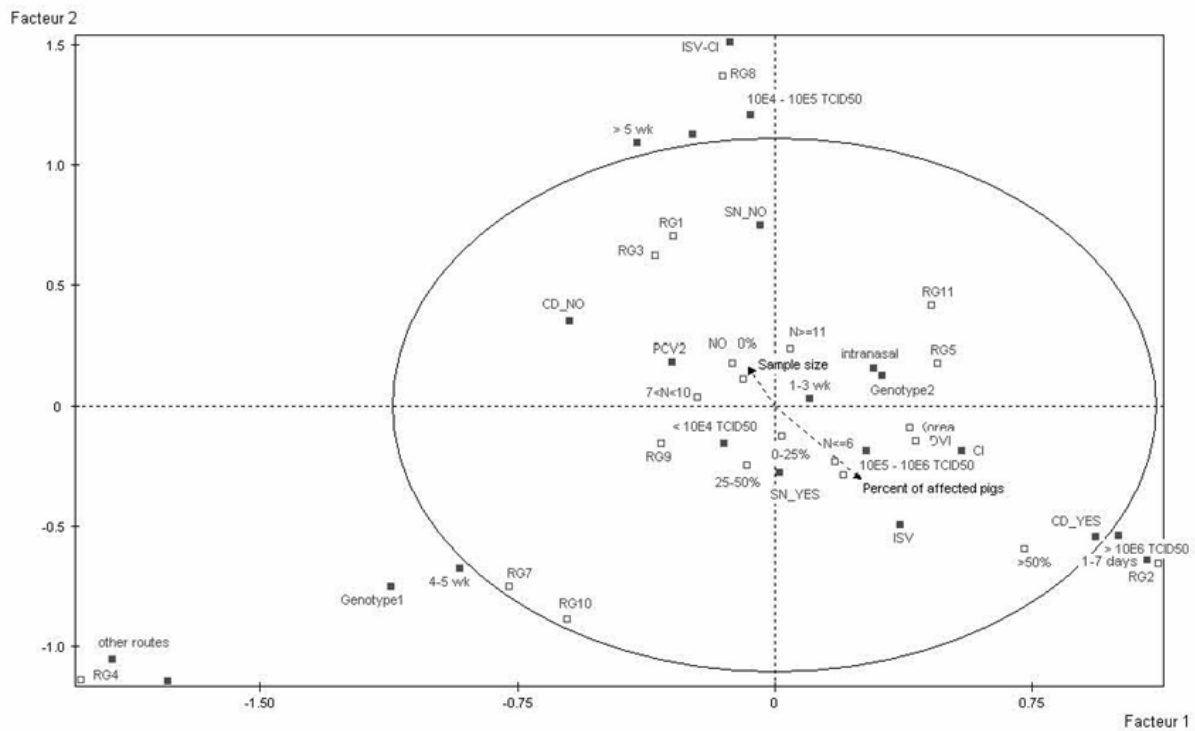
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694 **Figures**

695 Figure 1. Scatter of the multiple correspondence analysis. Modalities of each variable are
 696 represented in a two dimensional plane along the horizontal axis (first axis, inertia = 18.7%)
 697 and the vertical axis (second axis, inertia = 14.2%). Black squares correspond to nominal
 698 active variables: age at inoculation (1-7 days, 1-3 wk, 4-5 wk, >5 wk), colostrum deprivation
 699 (CD_YES, CD_NO), seronegative for PCV2 (SN_YES, SN_NO), route of inoculation
 700 (intranasal, other routes), dose of inoculum (<10E4 TCID50, 10E4-10E5 TCID50, 10E5-10E6
 701 TCID50, >10E6 TCID50), PCV2 genotype (Genotype1, Genotype2), type of experiment
 702 (PCV2: PCV2 alone, ISV: PCV2 + immunostimulator or vaccination, CI: co-infection, ISV-
 703 CI: combination of categories ISV and CI). White squares correspond to illustrative variables:
 704 percent affected (0%, 0-25 %, 25-50%, >50%), sample size (N≤6, 7<N<10, N≥11), research
 705 group (RG1 to RG12). Arrows indicate the direction of increase of quantitative variables
 706 (sample size and percent of affected pigs) along the factorial space.



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