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1 **Similar frequency of *Porcine circovirus 3* (PCV-3) detection in serum samples of**
2 **pigs affected by digestive or respiratory disorders and age-matched clinically**
3 **healthy pigs**
4
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15

16 **Summary**

17 *Porcine circovirus 3* (PCV-3) has been identified in pigs affected by different disease
18 conditions, although its pathogenicity remains unclear. The objective of the present
19 study was to assess the frequency of PCV-3 infection in serum samples from animals
20 suffering from postweaning respiratory or digestive disorders as well as in healthy
21 animals. A total of 315 swine serum samples were analysed for PCV-3 DNA detection
22 by conventional PCR; positive samples were further assayed with a quantitative PCR
23 and partially sequenced. Sera were obtained from 4 week- to 4 month-old pigs clinically
24 diagnosed with respiratory (n=129) or digestive (n=126) disorders. Serum samples of
25 age-matched healthy animals (n=60) served as negative control. Pigs with clinical
26 respiratory signs had a wide variety of pulmonary lesions including suppurative
27 bronchopneumonia, interstitial pneumonia, fibrinous-necrotizing pneumonia and/or
28 pleuritis. Animals with enteric signs displayed histopathological findings like villus
29 atrophy and fusion, catarrhal enteritis and/or catarrhal colitis. Overall, PCV-3 DNA was
30 detected in 19 out of 315 analysed samples (6.0%). Among the diseased animals, PCV-
31 3 was found in 6.2% (8 out of 129) and 5.6% (7 out of 126) of pigs with respiratory and
32 digestive disorders, respectively. The frequency of PCV-3 PCR positive samples among
33 healthy pigs was 6.7% (4 out of 60). No apparent association was observed between
34 PCR positive cases and any type of histopathological lesion. The phylogenetic analysis
35 of the partial genome sequences obtained showed high identity among viruses from the
36 three groups of animals studied. In conclusion, PCV-3 was present in the serum of
37 diseased and healthy pigs to similar percentages, suggesting that this virus does not
38 seem to be causally associated with respiratory or enteric disorders.

39

40 **KEYWORDS:** porcine circovirus 3 (PCV-3); polymerase chain reaction (PCR);
41 digestive disease; respiratory disease; healthy; pig
42

1. INTRODUCTION

Circoviruses are circular, non-enveloped, single-stranded DNA viruses belonging to family *Circoviridae*, genus *Circovirus* (Rosario et al., 2017). Three types of porcine circoviruses (PCVs) have been described so far, PCV-1, PCV-2 and PCV-3. PCV-1 is considered non-pathogenic for swine, PCV-2 is an important pathogen for swine production worldwide causing porcine circovirus diseases (PCVDs) and, finally, the recently described PCV-3 has not yet been characterized from a pathogenic point of view (Klaumann, Correa-Fiz et al., 2018).

PCV-3 was discovered in 2015 by next generation sequencing (NGS) methods in animals with various disease conditions, including digestive disorders, cardiac and multisystemic inflammation, and respiratory and neurological signs (Phan et al., 2016). At the same time, PCV-3 was detected in sows displaying reproductive problems, increased mortality rate, and suffering from respiratory disorders and porcine dermatitis and nephropathy syndrome (PDNS) (Palinski et al., 2017). Since the very first description of PCV-3, this virus has been found in pigs with respiratory clinical signs and lung lesions (Zhai et al., 2017; Kedkovid et al., 2018; Shen et al., 2018; Qi et al., 2019), digestive disorders (Zhai et al., 2017; Qi et al., 2019), congenital tremors (Chen et al., 2017), rectal prolapse (Phan et al., 2016) and periweaning failure-to-thrive syndrome (Franzo et al., 2019). However, several of these studies where PCV-3 was found in diseased animals failed to provide with healthy age-matched pigs as control groups (Kedkovid et al., 2018, Palinski et al., 2017, Phan et al., 2016, Shen et al., 2018).

The apparent most consistent relationship of this novel virus with disease occurrence is regarding respiratory and digestive disorders. Two studies found PCV-3 DNA in pigs with severe and/or mild respiratory disease compared to healthy animals, pointing out to a potential involvement of this virus in disease causation (Zhai et al., 2017;

68 Qi et al., 2019). Same studies also suggested a putative association with digestive
69 disorders based on the higher frequency of detection of the virus DNA in diseased animals
70 compared to healthy ones (Zhai et al., 2017; Qi et al., 2019).

71 Taking into account the information of the above described reports, the present
72 study was aimed to evaluate the putative association of PCV-3 infection in well-
73 pathologically characterized cases of pigs suffering from respiratory or digestive
74 disorders in comparison to age-matched clinically healthy animals.

75

76 ## 2. MATERIAL AND METHODS

77 ### 2.1 Samples

78 The study was performed with 255 swine serum samples obtained from animals
79 affected by digestive or respiratory disorders necropsied for diagnostic purposes. These
80 samples were stored at -20ºC at the serum bank of the Veterinary Pathology Diagnostic
81 Service at the Veterinary School of Barcelona. The selection criteria of these animals
82 were: 1) age (animals from 1-4 months of age (nursery and grower pigs), and 2) presence
83 of histopathological lesions at the respiratory (n=129) or enteric (n=126) tracts. A
84 negative control group of clinically healthy animals were selected for comparison
85 purposes. A total of 60 sera were retrieved from the serum bank of the *Centre de Recerca*
86 *en Sanitat Animal* (CReSA-IRTA), and consisted in a selection of necropsied (n=30) and
87 alive (n=30) 1-4 month-old pigs with no clinical signs and no lesions. The necropsied
88 animals belonged to control groups of different CReSA experimental inoculation studies.
89 The clinically healthy live pigs came from previous CReSA field trials. All selected
90 samples (n=315) were analysed for the presence of PCV-3 DNA.

91 Pigs affected by respiratory clinical signs **(**group R**)** were classified regarding
92 specific gross and/or microscopic findings (Table 1). Animals with interstitial pneumonia

(IP, n=30), suppurative bronchopneumonia (SBP, n=30), IP plus SBP (IP+SBP, n=30), pleuritis (P, n=19) and fibrinous-necrotizing pneumonia (FNP, n=20) were selected. Animals with digestive disorders **(**group D**)** were also classified by histopathological findings, including pigs with catarrhal enteritis (CE, n=55), CE with villi atrophy and fusion (CE+AFV, n=25) and catarrhal colitis (CC, n=46). The control group was composed of sera from 60 clinically healthy animals **(**group H).

2.2 DNA extraction, and conventional and quantitative PCR methods

DNA was extracted from 200 µL of serum using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol.

A conventional PCR targeting the *rep* gene region (ORF1) of PCV-3 was designed. Each reaction included 2.5 µL of extracted DNA, 12.5 µL of Go Taq® G2 Green Master Mix (Promega), 0.4 µM of each primer (forward (P1F) 5`-TTGTGGTGCTACGAGTGTCC-3` and reverse (P1R) 5`-CGTCTCCGTCAGAATCCGAG-3`), and sterile water at a final volume of 25 µL. Amplification was performed using the following thermal conditions: 5 min at 94˚C, followed by 40 cycles of 1 min at 94˚C, 1 min at 60˚C and 1 min at 72˚C, and a final elongation of 7 min at 72˚C. A full-length PCV-3 genome in a commercial plasmid (Klaumann, Dias-Alves et al., 2018) was used as positive control and sterile water as negative control. The PCR products (418 bp) were checked by electrophoresis on 1.5% TAE agarose gel.

A real time quantitative PCR (qPCR) to quantify the amount of viral DNA of conventional PCR positive samples was performed as previously described (Franzo, Legnardi, Centelleghe et al., 2018), with slight modifications. Briefly, 2 µL of extracted DNA was added to a mix of 1xDyNAmo Colour flash Probe qPCR mastermix (Thermo

118 Fisher Scientific), 0.6 µM and 0.3 µM of primers and probe targeting PCV-3 virus, 0.6
119 µM and 0.3 µM of primers and probe for internal control (IC), and 1 pg of IC plasmid
120 (Klaumann, Dias-Alves et al., 2018). The qPCR was performed with Applied
121 Biosystems® 7500 Real-Time machine with cycling parameters of 95˚C for 7 min,
122 followed by 40 cycles of 95˚C for 10s and 60˚C for 30s. Sterile water was used as negative
123 controls. A tenfold log dilution of the commercial plasmid mentioned above was used to
124 construct the standard curve. The qPCR results were expressed in copies of PCV-3
125 DNA/µL of serum.

126

127 **2.3 PCV-3 sequencing and phylogenetic studies**

128 Two different sequencing strategies based on *rep* gene and complete genome were
129 attempted.

130 Firstly, to partially sequence the *rep* gene (362 nt), the conventional PCR primers
131 were used; conditions for amplification included the utilisation of the DNA polymerase
132 Platinum™ SuperFi™ (Invitrogen™) kit and the thermal protocol of 2 min at 95˚C,
133 followed by 40 cycles of 30 s at 95˚C, 1 min at 60˚C and 1 min at 72˚C, and a final
134 elongation of 7 min at 72˚C. The amplicons were purified with a Kit NucleoSpin® Gel
135 extraction (Macherey-Nagel) according to the manufacturer's protocol and sequenced by
136 the Sanger method (ABI 3730XL - Macrogen Europe, Madrid, Spain). The quality of the
137 sequences was analysed by the Finch TV program and trimmed in BioEdit software 7.2.6
138 (Hall, 1999) together with all the sequences containing the same fragment available in
139 GenBank (247 sequences, accession date April 03rd, 2019). Clustal Omega (Sievers and
140 Higgins, 2014) was used to align and generate the percentage of nucleotide identity matrix
141 with the obtained sequences. Sequences were deposited at the NCBI GenBank with the
142 accession numbers MK904813 to MK904827.

143 Secondly, with the aim to sequence the full genome from PCV-3 PCR positive
144 samples, a rolling circle amplification (RCA) method was attempted to increase the
145 amount of PCR template using the ThempliPhi 100 amplification Kit (GE Healthcare) as
146 described by Fux et al. (2018). For the PCR, 3 µL of the 1:10 diluted RCA product was
147 used to amplify the complete PCV-3 genome using previously described primers (Fux et
148 al., 2018) and thermal conditions (Klaumann, Dias-Alves et al., 2018). The amplicons
149 were purified as described above and subjected to Sanger sequencing (ABI 3730XL -
150 Macrogen Europe, Madrid, Spain). The raw chromatograms were manually inspected
151 with Finch TV and the trimming was done with the BioEdit software 7.2.6 (Hall, 1999).
152 Partial PCV-3 genome sequence assembly was done with the different amplicons using
153 reference mapped-based strategy (Li and Durbin, 2010). The Integrative Genomics
154 Viewer (IGV) software (Robinson et al., 2017) was used for visualizing the assembly and
155 obtaining the consensus sequences for ORF1 and ORF2. Concatenated *rep* and *cap* genes
156 obtained were 1237 nt in length and were subsequently aligned with the 43 sequences
157 within the same genomic region previously used and classified by Fux et al. (2018) using
158 Muscle (Edgar, 2004) integrated in MEGA 7 (Kumar et al., 2016). A maximum likelihood
159 (ML) tree was constructed using the substitution model test with the lowest BIC score
160 (MEGA7). The Tamura-Nei model was used to build the phylogenetic tree, with
161 bootstrapping at 1000 replicates to analyse the robustness of the clustering. The tree was
162 edited using the iTOL program (Letunic and Bork, 2019). Sequences were deposited at
163 the NCBI GenBank with the accession numbers MK904828 to MK904831. The already
164 suggested PCV-3 subgrouping classification (PCV-3*a1*, PCV-3*a2,* PCV-3*b1* and PCV-
165 3*b2,* Fux et al., 2018) was done regarding the amino acid (aa) motifs from *Rep* and *Cap*
166 proteins.
167

2.4 Statistical analysis

The Clopper-Pearson method (Clopper and Pearson, 2006) was used to calculate the exact confidence interval (CI) at 95% level of confidence for the obtained PCV-3 PCR positive frequencies. The frequency of PCV-3 DNA detection among the different groups (R, D and H) and types of lesions within each group was compared with the Chi-square or Fisher´s exact tests using GraphPad software (GraphPad software Inc., San Diego, USA). *P* values <0.05 were considered statistically significant.

3. RESULTS

3.1 Detection of PCV-3

PCV-3 DNA was found in 19 out of the 315 studied samples (6.0%, 95% confidence interval, CI: 3.7-9.3). The frequency of detection was 6.2% (8 out of 129, 95% CI: 2.7-11.9) in pigs from group R and 5.6% (7 out of 126, 95% CI: 2.3-11.1) in pigs from group D. Viral DNA was also detected to a similar frequency in group H, being 4 out of 60 (6.7%, 95% CI: 1.8-16.2) PCV-3 PCR positive healthy pigs (Table 1). No statistical differences were observed among studied groups, and no association of any specific lesion with PCV-3 detection was found.

Conventional PCR positive samples were quantified by qPCR, except five samples that were below the quantification limit of the technique (between one and ten copies of DNA/µL). The PCV-3 load detected in the positive cases was low, with mean viral loads in the different groups of lesions ranging from $10^{0.5}$ to $10^{2.8}$ copies/µL of serum (Table 1).

3.2 Sequence and phylogenetic analysis

192 From the 19 PCR positive samples, 15 sequences from the *rep* gene fragment were
193 obtained. From these sequences, eight belonged to pigs from the group R, three from
194 group D and four from group H. The identity matrix comparing these 15 *rep* fragments
195 showed a percentage of identity ranging from 98.34 to 100%. When these fragments were
196 compared with the same genomic regions of 247 available GenBank reference sequences,
197 such percentage ranged from 91.99 to 100%.

198 Four sequences of 1237 nt in length (including the complete *rep* gene with 890 nt
199 and part from the *cap* gene with 347 nt) were obtained from four RCA-PCR positive
200 samples: two from samples belonging to group H (samples No. 409 and 441); one from
201 group R (sample No. 1099) and one from group D (sample No. 169). Based on the ML
202 phylogenetic tree, samples No. 409 and 1099 belonged to the proposed subgroup *a1*,
203 while samples No. 441 grouped into subgroup *b1* and No. 169 were from subgroup *b2*
204 (Figure 1).

205

206 **4. DISCUSSION**

207 The present study aimed to expand the current knowledge on PCV-3 respiratory
208 and digestive disease association based on a case-control study performed with clinical-
209 pathologically well-characterized pigs. Each analysed group corresponded to specific
210 pathological findings that are generically considered to be caused by bacteria (SBP, P,
211 FNP, CE, CC) or viruses (IP, AFV+CE) (Caswell and Williams, 2016; Uzal et al., 2016)

212 In contrast to the two previous studies evaluating such putative relationship (Zhai
213 et al., 2017; Qi et al., 2019), no significant differences in terms of PCV-3 DNA frequency
214 detection were found among pigs with respiratory or digestive disorders compared
215 between them or with age-matched healthy animals. The previously mentioned studies
216 referred to samples from China and used either serum (Zhai et al., 2017) as in the present

217 work or intestinal/lung tissues (Qi et al., 2019), collected based exclusively in the animals
218 showing clinical signs. The matrix (sample type) used for studying PCV-3 frequency of
219 infection may offer some variability (Phan et al., 2016; Klaumann, Dias-Alves et al.,
220 2018; Shen et al., 2018) and, therefore, may partly explain differing results between the
221 present study and that of Qi et al. (2019). However, the same potential explanation would
222 not apply to the other study (Zhai et al., 2017), since serum was also used from affected
223 and non-affected pigs.

224 The two abovementioned works studying the frequency of PCV-3 in pigs with
225 digestive disorders also included control animals. Specifically, Qi *et al.* (2019) used
226 intestinal tissue samples from 480 pigs with acute diarrhoea and/or vomiting and 42
227 faeces of healthy pigs; whereas 50 out of 480 samples (10.4 %) were PCV-3 PCR positive,
228 all 42 healthy pigs were found to be negative. On the other hand, Zhai *et al*. (2017) found
229 17.14 % (6/35) PCR positive serum samples from pigs with diarrhoea and 2.86 % (1/35)
230 in animals without clinical signs. Both studies also found significant differences when
231 comparing pigs affected by respiratory problems and healthy ones. Indeed, Qi et al.
232 (2019) found 26.6% (25/94) and 0% (0/42) PCV-3 frequencies in diseased and healthy
233 animals, respectively. Similarly, Zhai et al. (2018) found a PCV-3 PCR positivity of 29%
234 (split as 63.75%, 51/80, in pigs with severe respiratory disease and 13.14%, 23/175, in
235 animals with mild signs) and 1.9% (4/216) in healthy pigs, respectively. In another study,
236 pigs with respiratory disease complex (PRDC) from Thailand showed significantly higher
237 PCV-3 detection rate (60%, 15/25) in serum compared to healthy pigs (28%, 7/25)
238 (Kedkovid et al., 2018). The apparently contradictory results offered by the present study
239 may represent different epidemiological situations in different countries and/or different
240 sensitivity of PCR techniques used.

241 Noteworthy, animal selection criteria from previous studies were mainly based on
242 clinical signs. For this reason, the present work split the clinical conditions by observed
243 lesions in each case. This selection is important, since lesions like IP and AFV+CE are
244 generally attributable to viral infections (Caswell and Williams, 2016; Uzal et al., 2016),
245 but no higher frequency of PCV-3 detection was found in any group of animals displaying
246 these pathological findings. In consequence, and supported by the fact that very low viral
247 loads were found in analysed animals, obtained results do not point out to PCV-3 as a
248 likely cause of these lesions. In contrast, two of the abovementioned Asian studies (Zhai
249 et al., 2017; Kedkovid et al., 2018) indicated that animals displaying respiratory clinical
250 signs had higher viral load than healthy ones. In the study from Zhai et al. (2017), pigs
251 with severe respiratory disease showed Ct values lower than 30 while the ones with either
252 mild respiratory disease or healthy animals showed Ct values higher than 25 or 30,
253 respectively (Zhai et al., 2017). The sick pigs from Kedkovid et al. (2018) study presented
254 titres of 3.2 ± 2.8 log genomic copies/mL, while healthy ones had lower titres (1.6 ± 2.6
255 log genomic copies/mL). In all cases, it would have been interesting to assess the type of
256 lesions those diseased animals displayed in these studies.
257 The relatively low frequency of PCV-3 DNA detection in all pig groups fits
258 relatively well with a previous retrospective study performed in Spain (Klaumann, Franzo
259 et al., 2018) where 11.4% (75 out of 654) of PCV-3 serum samples were found PCR
260 positive. Nevertheless, and based on existing literature, the prevalence of PCV-3 infection
261 seems to be very variable (Klaumann, Correa-Fiz et al., 2018). This fact makes difficult
262 to establish if such differences are due to a real variability among countries or simply
263 reflect the frequency of a limited number or type of samples examined in each study.
264 The phylogenetic analysis of all obtained partial sequences (the ones with 362 nt
265 as well as those with 1237 nt) showed high percentage of nucleotide identity compared

266 to the publicly available sequences, as it has been already published for this virus (Zheng
267 et al., 2017; Fux et al., 2018; Franzo, Legnardi, Hjulsager et al., 2018; Zhou et al., 2018).
268 Although Fux et al. (2018) proposed different subtypes for PCV-3, the very high
269 nucleotide identity of available sequences for this virus do not suggest a potential relation
270 with the clinical or pathological outcome; in fact, no clustering of the obtained sequences
271 was observed regarding the disease status.

272 In summary, PCV-3 DNA was found to similar percentages in pigs affected by
273 both respiratory and enteric disorders as well as in age-matched healthy animals.
274 Moreover, no apparent association was found between the presence of viral genome in
275 serum and particular lesions generally attributed to viral infections in both respiratory and
276 digestive tracts. Therefore, the results obtained throughout this study do not support a
277 potential association of PCV-3 with respiratory or enteric disease occurrence.
278 Definitively, further studies are needed to elucidate the putative association of PCV-3
279 with different pathological outcomes.

280

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287

288 **6. CONLFICT OF INTEREST STATEMENT**

289 All authors have declared no conflict of interest.

290

291 **7. REFERENCES**

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379 Figure legend

380

381 **FIGURE 1.** Phylogenetic analysis of PCV-3 based on partial genome (1237 nt). The
382 tree was constructed using Maximum likelihood method (Tamura-Nei model) at 1000
383 bootstrap. Circles in branches represent bootstrap values with sizes proportional to the
384 bootstrap value (only values higher than 50 are shown). Sequences obtained in the
385 present study are labelled in red. Colours indicate the subtypes of PCV-3 suggested by
386 Fux et al. (2018).